[Personal information and report codes redacted]

Application to FSANZ for the Inclusion of Z6 and V11 Potatoes with late blight protection, low free asparagine, lowered reducing sugars and reduced blackspot in Standard 1.5.2 Food Produced Using Gene Technology

Submitting Company:
SPS International Inc.

1099 West Front Street, Boise, ID 83702, United States of America

Submitted By:
[Personal information redacted]

Contributing Authors:
[Personal information redacted]

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SPS International Inc.

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SPS-∅∅∅Z6-6 **SPS-∅∅V11-6**

***Corresponding Author:**
[Personal information redacted]
SPS International Inc.
1099 West Front Street
Boise, ID 83702
United States of America

Tel.: ([Personal information redacted]
E-mail: [Personal information redacted]

This document does not contain confidential information

Part 1 General Requirements (3.1.1)

A. Executive Summary

SPS International, Inc. (SPSII) has pioneered a new approach that uses Innate® technologies to transform potato plants with potato genomic DNA, without the incorporation of selectable markers or vector backbone sequences. SPSII has developed the Snowden event SPS-∅∅∅Z6-5, hereafter referred to as event Z6. This event was developed to address the needs of the potato growers, industry and consumers for potatoes with late blight protection, low free asparagine, lowered reducing sugars and reduced black spot.

The event Z6 was developed by transforming the potato variety Snowden with pSIM1278 and pSIM1678. FSANZ has previously received submissions from SPSII for potato events transformed with pSIM1278 and pSIM1678: FSANZ Application Number A1128 and A1139. FSANZ has not identified any public health and safety concerns in its assessment of the potato events.

The Z6 event was developed by transforming Snowden with pSIM1278 (Event V11) and subsequently transforming V11 with pSIM1678. Events similar to V11 and Z6, containing an insert either from pSIM1278 or both pSIM1278 and pSIM1678, have previously been assessed and authorised by the U.S. and Canadian regulatory agencies, including USDA, FDA, Health Canada, Canadian Food Inspection Agency and FSANZ.

The T-DNA of pSIM1278 contains DNA sequences intended to down regulate endogenous enzymes through the mechanism of RNA interference (RNAi). The sequences were chosen from genes of enzymes present in potato tubers:

- *Asn1* (asparagine synthetase) for reduced free asparagine, contributing to low acrylamide potential;
- *R1* (water dikinase) for lower reducing sugars, contributing to low acrylamide potential;
- *PhL* (phosphorylase-L) for lower reducing sugars, contributing to low acrylamide potential; and
- *Ppo5* (polyphenol oxidase-5) for reduced black spot.

The pSIM1678 T-DNA contains the late blight resistance gene *Rpi-vnt1.* Late blight, caused by the oomycete *Phytophthora infestans* (*P. infestans*), is a serious disease of potatoes. The *Rpi-vnt1* gene produces the VNT1 resistance protein (R-protein), found in the wild *Solanum* species *Solanum venturii* and *Solanum phureja*, which protects against foliar late blight. The VNT1 protein does not have a pesticidal mode of action, but rather enables the potato plant to detect a *P. infestans*-specific effector, Avr-Vnt1, and initiate the plant's native immune response. In addition, the T-DNA of pSIM1678 contains potato vacuolar invertase (*VInv*) DNA sequence designed to down regulate the potato vacuolar invertase enzyme though RNAi, resulting in lower reducing sugars.

The Z6 event with the desired modified traits was characterised and is the subject of this submission. In addition, SPSII asks that the *Australia New Zealand Food Standards Code* be amended to include event V11, which is the primary event for Z6.

The levels of free amino acids, reducing sugars, and PPO activity were measured as an assessment of trait efficacy. These results demonstrated that Z6 has the expected phenotype. The changes to levels of free amino acids and reducing sugars are not nutritionally consequential as they do not affect the levels of essential amino acids or other key nutrients important to potato (OECD, 2002). The significantly lower levels of free asparagine and reducing sugars resulted in lower acrylamide in fries and chips made from Z6 tubers. Additionally, the efficacy testing for PPO down regulation confirmed that PPO activity was significantly reduced in Z6 tubers, consistent with effective down regulation of PPO in each event.

Molecular characterisation of the event was performed to determine the number of copies, arrangement, and stability of the inserted DNA from both vectors. The event was confirmed to be free of *Agrobacterium*-

derived backbone DNA. In the United States, confined field trials were undertaken, with the conventional variety and other cultivated varieties used as controls. Results from these trials confirmed no changes were observed that could have an impact on the environment or affect genetic stability. Compositional analysis was performed on field-grown tubers to compare nutritional and anti-nutritional compounds and showed no biologically relevant differences existed that could result in increased risk to humans or other non-target organisms. Analysis of the VNT1 protein and putative polypeptides produced from the inserted DNA indicated there are no sequences with significant homology to known allergens or toxins in these Z6 potatoes.

Analysis of the event Z6 has not revealed any biologically relevant differences compared to the conventional variety, except for the intended late blight protection, low free asparagine, low reducing sugars, and low polyphenol oxidase activity. Collectively, results of the molecular characterisation, agronomic assessment, and composition analysis support this application for amendment to the *Australia New Zealand Food Standards Code* to allow inclusion of the Innate® potato event Z6 as well as the primary event V11 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

B. Applicant Details

(a)	Applicant’s name/s	[Personal information redacted]
(b)	Company/organisation name	SPS International, Inc.
(c)	Address (street and postal)	1099 West Front Street, Boise, ID 83702, USA
(d)	Telephone number	[Personal information redacted]
(e)	Email address	[Personal information redacted]
(f)	Nature of the applicant’s business	SPS International, Inc. primarily files applications for approval of Simplot biotech events in international markets.
(g)	Details of other individuals, companies or organisations associated with the application	[Personal information redacted] [Personal information redacted]

C. Purpose of the Application

This application seeks to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of potato events Z6 and V11 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

SPSII has developed and tested potato events that have reduced expression of five enzymes and express a wild potato gene that conveys foliar protection against late blight. The potato event described in this application has the unique OECD code: SPS-∅∅∅Z6-5 and is referred to as Z6 in this submission. SPSII currently does not intend to import the potato event into Australia or New Zealand. The primary aim of this application is to obtain a food safety approval to protect international trade. This submission is consistent with SPS membership in the Excellence Through Stewardship® (ETS) program, adhering to stewardship and industry best practice by obtaining regulatory approvals in production and import markets.

In addition, SPSII asks that the *Australia New Zealand Food Standards Code* be amended to include V11, which is the primary event for Z6. The molecular characterisation of the primary event will be reviewed as part of the Z6 safety review. Event V11 contains only the quality traits of one insert from plasmid pSIM1278 and does not have the late blight protection or the lower vacuolar invertase traits of the second insert, from plasmid pSIM1678. To facilitate this decision, the unique OECD code for the V11 event is: SPS-∅∅V11-6. The insertion is stable, and composition data for V11 has been included [unpublished report]. Event V11 has limited commercial importance and the primary purpose for obtaining approval is to protect international trade.

D. Justification for the Application

SPS International, Inc. has developed a new potato event, Z6. The new potato event was created using inserts containing potato DNA sequences that confer lower levels of free asparagine and reducing sugars, which together contribute to reduced acrylamide potential, and lower levels of polyphenol oxidase which reduces black spot. The event also contains a wild potato gene that confers late blight protection.

Late Blight Protection: Late blight caused by the oomycete *Phytophthora infestans*, is a devastating disease among cultivated *Solanaceae* species. If left untreated, late blight affects potato foliage and tubers causing rapid necrosis and crop loss. The Irish potato famine was the result of late blight and illustrates the destructive nature of the disease. The cultivation of event Z6 will enable growers to use an improved disease control strategy including integrated pest management (IPM) to manage late blight effectively.

Reduced Black Spot in Potatoes: Black spot refers to the black or greyish colour that may form in damaged or cut potatoes. It is a post-harvest physiological effect resulting from the handling of potato tubers during harvest, transport, processing, and storage and it contributes to waste experienced by growers, consumers, and processors. The enzymatic discoloration is associated with polyphenol oxidase (PPO) and occurs when the enzyme leaks out of the plastids of potatoes (Vaughn et al., 1988). Potatoes with black spot are either trimmed or rejected before processing, resulting in quality control challenges and economic loss. Lowering PPO levels in potatoes reduces the occurrence of black spot and this reduces grower, consumer, and processor waste.

Reduced Acrylamide Potential: Lowering the acrylamide potential of potatoes is important because acrylamide presents a potential health risk for consumers (FDA, 2013). Although acrylamide is not present in fresh potatoes, it forms in carbohydrate-rich foods when the amino acid asparagine and the reducing sugars, glucose and fructose, are heated at temperatures above 120 °C (O'Brien and Morrissey, 1989). Lowering the concentrations of free asparagine, glucose, and fructose in potatoes reduces the acrylamide potential of cooked potatoes. The biochemical basis of acrylamide formation has been published by Stadler, 2005.

Potatoes with lower acrylamide address a potential health concern for consumers, especially in light of recent toxicology studies (Food Drink Europe[1], 2014; Health Canada, 2012; NTP, 2012). Various governments have responded to the findings of acrylamide in food by providing guidance documents and conducting surveys to assess the source and intake of acrylamide in foods (EFSA CONTAM Panel, 2015; FDA, 2006; Food Drink Europe, 2014).

The United States FDA has proposed guidance for industry on the reduction of acrylamide levels in food products (FDA, 2013). In their *Draft Guidance for Industry on Acrylamide in Foods*, the FDA notes "Reducing acrylamide in foods may mitigate potential human health risks from exposure to acrylamide." An extensive list of potential mitigation techniques were summarised in the guidance document (FDA, 2013), focusing primarily on the reducing sugar levels in potatoes. Many of the methods in FDA's Guidance document are consistent with those reported in the *Acrylamide Toolbox* published by Food Drink Europe (Food Drink Europe, 2014).

Estimated dietary exposures of Australian consumers to acrylamide in food were investigated as a part of the first phase of the 24th Australian Total Diet Study (FSANZ, 2014). The study found that the levels of acrylamide were generally lower than, or comparable to, those reported in previous Australian and international studies. However, the estimated dietary exposures of Australian consumers were in the range considered to be of possible concern to human health by the Joint Expert Committee on Food Additives.

In New Zealand, the Ministry for Primary Industries (MPI) reassessed dietary exposure with a survey of foods contributing to acrylamide intake in New Zealand (Cressey et al., 2012). The survey found that dietary exposure estimates have remained fairly constant since a previous survey in 2006.

International food regulators are working with industry to reduce acrylamide levels. New farming and processing techniques are being investigated to produce lower levels of acrylamide, for example, lowering cooking temperatures, using enzymes that reduce acrylamide formation, and obtaining raw materials with lower reducing sugar levels.

FSANZ is encouraging and supporting industry to use enzymes that reduce acrylamide formation and urging industry to adopt an *Acrylamide Toolbox* produced by the Confederation of the Food and Drink Industries of the EU (CIAA, 2013). A Codex working group has created a Code of Practice for reducing acrylamide in food (Codex, 2009). Both FSANZ and MPI contributed to the development of this Code of Practice. The Z6 event satisfies these recommendations by providing a product with lower levels of reducing sugars and free asparagine. These characteristics result in significantly lower levels of acrylamide in processed potato products.

E. Information to Support the Application

This application consists of 2 parts containing information in accordance with the following checklists:

- Part 1: General requirements (3.1.1)
- Part 2: Foods produced using gene technology (3.5.1) main document, Part 2 information. Supplement form molecular analysis.

[1] Food Drink Europe, 2014 (http://www.fooddrinkeurope.eu/publications/category/toolkits/)

F. Assessment Procedure

SPSII is anticipating that this application will be considered under the **General Procedure** for Administrative Assessment process by Food Standards Australia New Zealand.

G. Confidential Commercial Information (CCI)

Confidential Commercial Information (CCI) has not been included in this submission document.

Release of Information

SPSII is submitting the information in this application for review by Food Standards Australia New Zealand (FSANZ) for amendment to the Food **Standard 1.5.2** Food Produced Using Gene Technology. SPSII holds proprietary rights to the extent allowable by law to all such information and by submitting this information, SPSII does not authorise its release to any third party except to the extent it is duly requested under the Freedom of Information Act 1982 (*FOI Act*) or in compliance with the responsibility of FSANZ to publish documents required under Sections 8, 8(A), 8(C) and 8(D) of the *FOI Act*; and this information is responsive to the specific aforementioned request. Accordingly, except as specifically stated above, SPSII does not authorise the release, publication or other distribution of this information (including website posting or otherwise), nor does SPSII authorise any third party to use, obtain, or rely upon this information, directly or indirectly, as part of any other application or for any other use, without SPSII's prior notice and written consent. Submission of this information does not in any way waive SPSII's rights (including rights to exclusivity and compensation) to such information.

H. Other Confidential Information

No additional confidential material is included in this submission document.

I. Exclusive Capturable Commercial Benefit

SPSII acknowledges that the proposed amendment to the Standard will likely result in an exclusive capturable commercial benefit being accrued to the parent company (J.R. Simplot Company) as defined in Section 8 of the *FSANZ Act*.

Costs and Benefits

Today, one of the main global challenges is how to ensure food security for a growing population whilst ensuring long-term sustainable development. According to the FAO, food production will need to grow by 70% to feed world population, predicted to reach 9 billion by 2050 (Alexandratos and Bruinsma, 2012). Current trends, such as increasing urban population, shift of lifestyle and diet patterns of the rising middle class in emerging economies, along with climate change, put considerable pressure on the earth's resources.

In the meantime, while food insecurity remains unacceptably high, each year, massive quantities of food are lost worldwide due to spoilage and infestations on the journey from farm to consumers. One of the major ways of strengthening food security is by reducing these post-harvest losses.

The term 'post-harvest loss' refers to measurable quantitative and qualitative food loss in the post-harvest supply chain. The supply chain comprises interconnected activities from the time of harvest through crop processing, marketing and food preparation, to the final decision by the consumer to eat or discard the food.

Post-harvest loss reduction interventions are a critical component of efforts to reduce food insecurity, as part of an integrated approach to realising agriculture's full potential to meet the world's increasing food and energy needs. Therefore, reducing post-harvest loss by making more effective use of today's crops, improving productivity on existing farmland, and bringing additional acreage into sustainable production, is critical to facing the challenge of feeding an increasing world population.

As a global staple food crop, post-harvest losses in the potato supply chain due to black spot, enzymatic darkening, cause waste and economic loss. These issues occur in the fresh and processed food supply chains in both industrial and third world countries across the globe.

Enzymatic darkening is a widespread colour reaction occurring in fruits and vegetables, which involves the interaction of oxygen, phenolic compounds and polyphenol oxidases (PPOs). Darkening is usually initiated by bruising of the potato caused by impact and pressure during harvest and storage. It also is initiated by slicing/dicing/juicing fresh fruit and/or vegetables for use in fresh consumption or as part of preparation for further processing. As a result, PPO catalyses the enzymatic oxidation and conversion of monophenols to o-diphenols and o-dihydroxyphenols to o-quinones. The quinone products polymerise and react with amino acid groups of cellular proteins, resulting in black or brown pigment deposits ('darkening').

A variety of fruits and vegetables, such as apple, pear, banana, peach, lettuce and potato, are especially susceptible to enzymatic darkening during storage and processing. Darkening has a negative effect on appearance and may impair other sensory properties including taste, odour and texture.

The Z6 event uses RNAi gene silencing technology to regulate the expression of the genes responsible for the enzymatic darkening process. As a result, Innate® potatoes are less susceptible than conventional potatoes to darkening and the onset of black spot from bruising caused by impact and pressure during harvest, storage and food preparation.

Potatoes with black spot typically are trimmed or discarded before processing, resulting in both quality control challenges and economic loss. One study suggests that 1.9 million metric tons of bruise losses occurred at the grower, packer, retailer and foodservice levels of the market chain in the United States in 2013 (Halterman et al., 2016). Another study estimates that 35% of fresh potatoes were lost as food waste at the retail and consumer levels of the market chain in the United States in 2008 (Buzby et al., 2011); a significant portion of these losses would be associated with black spot. Reducing PPO levels in potatoes decreases the occurrence of black spot, resulting in increased tuber quality and less food waste (Halterman et al., 2016). In Y9, reduced black spot was achieved by down regulating PPO in tubers.

Research has demonstrated potential for the following benefits to be captured by United States potato farmers, supply chain participants and consumers following the introduction of Innate® technology in a range of potato varieties.

- Innate® potatoes reduce bruise and black spot up to 44% compared to conventional varieties.
- Using Innate® technology, it is possible for packers to experience an estimated 15% increased pack-out of fresh-grade potatoes, providing better utilisation, improved processing efficiencies and less waste.
- Because Innate® potatoes show less bruise, there will be fewer rejected loads by processors and a reduction in price discounts based on quality downgrades due to bruising.
- Consumers will throw away fewer fresh potatoes – it is estimated that up to 35% of fresh potatoes are wasted in the United States alone, representing 1.67 billion kg per year which has been estimated to cost upwards of $1.7 billion annually (Buzby et al., 2011) United States Journal of Consumer Affairs.
- Food approvals in influential countries like Australia increase the likelihood that these potatoes can become available in developing countries to help alleviate hunger in resource-scarce parts of the world. Simplot is working to help ensure this can happen by partnering with Michigan State University to develop late blight protected varieties for Bangladesh and Indonesian farmers. In 2019 Simplot

provided late blight protected events to Michigan State University for field efficacy testing in these two countries. These events have three R-genes for durable late blight protection and are in two farmer preferred varieties chosen by Bangladesh and Indonesia.This initiative is part of the United States Government's Feed the Future initiative.

In addition to reduced black spot, the Z6 event has lower levels of free asparagine and reducing sugars, which decreases the potential for acrylamide formation. Acrylamide is a chemical compound that occurs when potatoes, wheat, coffee, and other foods are cooked at high temperatures. The United States FDA and the European Food Safety Authority have classified acrylamide as a probable carcinogen. Many international and national regulatory agencies advise limiting dietary intake of acrylamide.

Most potatoes consumed in Australia are grown domestically. Domestic production of potato in Australia (2017/2018 – 1.4 million metric tonnes over 28,000 ha)[2] is supplemented by imports of processed potato, predominantly from the United States. Australian fresh potato exports were 46,924 tonnes for the year ending June 2018. This was made up of 36,529 tonnes fresh product and 10,395 tonnes processed product. Of the total domestic production, 66% is sent to processing, 32% is sold fresh while the remaining is sent for fresh export.

Potatoes enjoy consistent market penetration year-round at 52-58%, confirming their role as a staple Australian product on both summer and winter menus. Family households typically buy more potatoes at each purchase than smaller households, while households with lower discretionary income are more likely to purchase lower priced pre-packed products compared to those with higher discretionary income (AusVeg[3], August 2015).

Domestic production in New Zealand (2017/2018 – 527,190 metric tonnes over 10,344 ha)[4] is valued at $NZ 1 billion per annum. New Zealand exports were 99,009 metric tonnes for the year ending 2018. Of the total domestic production, 67.22% is sent for processing, 28.6% is sold as fresh/table and the remaining 4% is seed for the following season.

In both Australia and New Zealand, consumer research has confirmed that all major uses of potato involve some level of cooked preparation. The versatility of potato has led to a range of uses, with the majority being centred around dinner, and to lesser degree lunch time occasions. Seasonal influences have little bearing on the main uses of potato, reflecting patterns of consistent consumption year-round. Within the Australian and New Zealand diets, potatoes are prepared and consumed in the following forms:

- Boiled, Microwaved, Steamed;
- Deep fried;
- Mashed;
- Roasted;
- Baked/Grilled;
- Salad – cooked;
- Soup/Sauce;
- Stir fry;

[2] The Australian Horticulture Statistics Handbook 2017/18 (AH15001): retrieved December 2019 from https://www.horticulture.com.au/growers/help-your-business-grow/research-reports-publications-fact-sheets-and-more/australian-horticulture-statistics-handbook/

[3] Ausveg's Potato Consumer Research http://ausveg.com.au/potatoes/potato-consumer-research.htm.

[4] Potatoes New Zealand Inc. AGM Papers and Annual Report 2019: retrieved December 2019 from https://potatoesnz.co.nz/administration/annual-reports/

- Juiced;
- Sandwich/burger/wrap; and
- Snacks – potato chips / straws.

In Australia, for the year ending June 2014, per capita consumption for fresh potatoes purchased through retail for home consumption was 14.56 kg, and for fresh potatoes purchased and consumed in food service away from home was 3.24 kg. Per capita consumption for processed potato products purchased through retail for home and foodservice consumption was 17.73 kg. Therefore, total annual consumption was 35.53 kg per capita (Fresh Logic, 2014). In New Zealand, average consumption was estimated at 22.89Kg per capita (Potatoes NZ, 2014).

The importance of potato and the impact of black spot darkening in the fresh potato market is demonstrated in Ausveg's Potato Consumer Research of August 2015 that reported that - "Consumers are concerned about wastage and are seeking information on freshness (best before dates). This could be in the form of providing estimated freshness for loose potatoes at the point of sale, such as 'will last for 2 weeks in your cupboard'."

The event Z6 also contains the *Rpi-vnt1* gene from *S. venturii* that confers foliar protection to an important potato pathogen, *P. infestans*, the causal agent of late blight (Foster et al., 2009). Late blight is a devastating disease among cultivated *Solanaceae* species. In potato, late blight affects foliage and tubers causing rapid necrosis and crop loss if left untreated (Haverkort et al., 2008). The Irish potato famine was the result of late blight and demonstrates the destructiveness of the disease (FAO, 2008). Symptoms appear at first as water-soaked spots on leaves. Inside the infected leaves, sporangiophores emerge through the stomata of the stems and leaves and produce sporangia. The sporangia when ripe become detached, and are easily spread by wind and rain causing new infections (Agrios, 2004). During irrigation or wet weather conditions, sporangia are washed down from the leaves and into the soil where they subsequently penetrate and infect the tubers (Agrios, 2004). Infected tubers will likely rot in storage, contaminate equipment, and spread infection to non-infected tubers (Miller et al., 2006). Under favorable conditions for late blight (i.e. humidity, temperature, and host susceptibility), asexual reproduction of the pathogen occurs in as few as four days leading to disease epidemics (Agrios, 2004).

Nine of the top ten registered seed varieties grown in Canada are susceptible to late blight, comprising approximately 66% of planted acreage (CFIA, 2015; NPC, 2015). Therefore, IPM practices to control late blight rely primarily on chemical fungicides, crop rotation, and other cultural controls to minimize risk of infection (Canadian Horticulture Council, 2011; Miller et al., 2006). The Pest Management Centre of Agriculture and Agri-Food Canada recommend both a prophylactic spray fungicide program and field rotations with crops such as cereals and forages in order to minimize the occurrence of disease and improve soil and crop productivity (Agriculture and Agri-Food Canada, 2005). The planting of late blight resistant varieties allows the use of a balanced disease control strategy that employs IPM programs while maintaining durability of the trait.

Resistance genes (R-genes) in wild *Solanum* species provide natural protection to late blight (Sedlák et al., 2005). These genes can be bred or introduced into new varieties that then become resistant to late blight. Potato breeding programs from the mid-twentieth century used *Solanum demissum*, a wild potato species found in central Mexico and a good source of protection against certain strains of late blight (Pel, 2010; Vleeshouwers et al., 2011). *S. demissum* R-genes designated *R1-R11* have been identified, some of which have been widely used for introgression in European breeding programs to help control late blight (Malcolmson and Black, 1966).

Numerous research studies have been carried out worldwide to develop late blight resistant potato cultivars and improve their durability. The introduction of new cultivars containing these R-genes was initially successful, but rapidly evolving populations of *P. infestans* reduced their efficacy (Fry et al., 2015). Durability in the field of a particular R-gene is variable (Leach et al., 2001), and additional novel resistance genes against

P. infestans are being discovered from other wild *Solanum* species. Genes from wild *Solanum* species have been integrated into many edible potato cultivars through breeding with wild species such as *S. bulbocastanum* (Park et al., 2005), S*. stoloniferum* (Haverkort et al., 2008; Hutten et al., 2013), *S. microdontum* (Tan et al., 2008) and *S. phureja* (Śliwka et al., 2013).

Potatoes are highly heterozygous and subject to inbreeding depression, so many years are required to develop new varieties through conventional breeding. Due to these challenges, potato varieties do not have a high frequency of introduction and discontinuation. Because it is difficult to breed and backcross traits into potatoes, biotech offers a mechanism to modify traits while maintaining desired traits in a variety.

The potato industry is a substantial and important industry across both Australia and New Zealand. Potatoes are the highest value horticultural crop grown for consumption in Australia and are grown in all states of Australia except the Northern Territory. In New Zealand, potatoes are grown in Pukekohe, Waikato, Hawkes Bay, Manawatu, Canterbury and Southland.

The Australian National Potato Industry Biosecurity Plan (the Potato Biosecurity Plan) was developed by Plant Health Australia (PHA) in collaboration with industry and government stakeholders and was launched in May 2007 and updated in 2013. The Potato Biosecurity Plan notes that Australia's geographic isolation and lack of shared land borders have, in the past, provided a degree of natural protection from exotic threats.

The Potato Biosecurity Plan lists Late blight – A2 mating type – *Phytophthora infestans*, as one of the top-ranked pest threats to the Australian potato industry. The event Z6 is currently not intended for the Australian market, however, such technology represents future opportunities for Australian growers and consumers.

J. International and Other National Standards

Applications for approval of SPS-∅∅∅Z6-5 and SPS-∅∅V11-6 have been submitted to other jurisdictions (Table 1).

Responsible environmental stewardship and deployment of biotechnology-derived products are important to SPS International, Inc., to its parent company the J.R. Simplot Company, and to Simplot Plant Sciences (SPS), the biotechnology group within the J.R. Simplot Company. SPS is a member of Excellence Through Stewardship® (ETS), an industry-coordinated initiative that promotes the global adoption of stewardship programs and quality management systems for the full life cycle of biotechnology-derived plant products. The ETS "*Guide for Product Launch Stewardship of Biotechnology-Derived Products*" (ETS, 2013) also references and is consistent with the product launch policies of the Biotechnology Industry Organisation and Crop Life International.

Table 1: Current Applications and Approval Status for SPS-∅∅V11-6, and SPS-∅∅∅Z6-5

Country	Competent National Authority	Type of Authorisation	Approval Status	
			V11-6	Z6-5
United States	United States Department of Agriculture (USDA)	Determination of non-regulated status	Approved	Under Review
	Food and Drug Administration (FDA)	Food and feed safety assessment	Approved	Under Review
	Environmental Protection Agency (EPA)	Permanent exemption from Tolerance to VNT1; Event Registration	Not applicable	Under Review
Canada	Canadian Food Inspection Agency (CFIA)	Unconfined environmental release	Approved	Retransformation review underway
		Use in livestock feed	Approved	Retransformation review underway
	Health Canada	Food approval	Approved	Under review

[a] Event V11 has not been commercialised

The Codex *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (CAC/GL 45-2003, 2006) is applicable to the assessment of this application to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of event SPS-∅∅∅Z6-5, and its primary event SPS-∅∅V11-6 in Standard 1.5.2–Food Produced Using Gene Technology.

Simplot notes: that the *Codex Guideline* does not make allowances for familiarity with risk, such as occurs with events resulting from the retransformation of different varieties of a vegetatively reproduced crop.

K. Statutory Declaration – Australia

[Personal information redacted]

L. Checklists Provided With Application

General Requirements

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
☒	2	A Form of application ☒ Application in English ☒ Executive Summary (separated from main application electronically) ☒ Relevant sections of Part 3 clearly identified ☒ Pages sequentially numbered ☒ Electronic copy (searchable) ☒ All references provided
☒	3	B Applicant details
☒	4	C Purpose of the application
☒	4	D Justification for the application ☒ Regulatory impact information ☒ Impact on international trade
☒	5	E Information to support the application ☒ Data requirements
☒	6	F Assessment procedure ☒ General ☐ Major ☐ Minor ☐ High level health claim variation
☒	6	G Confidential commercial information ☐ CCI material separated from other application material ☐ Formal request including reasons ☐ Non-confidential summary provided
☒	6	H Other confidential information ☐ Confidential material separated from other application material ☐ Formal request including reasons
☒	6	I Exclusive Capturable Commercial Benefit ☒ Justification provided
☒	10	J International and other national standards ☒ International standards ☒ Other national standards
☒	12	K Statutory Declaration
☒	13	L Checklist/s provided with application ☒ 3.1.1 Checklist ☒ All page number references from application included ☒ Any other relevant checklists for Chapters 3.2–3.7

Foods Produced Using Gene Technology

Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
☒	23	A.1 Nature and identity
☒	24	A.2 History of use of host and donor organisms
☒	30	A.3 Nature of genetic modification
☒	72	B.1 Characterisation and safety assessment
☒	86	B.2 New proteins
☒	87	B.3 Other (non-protein) new substances
☐	N/A	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
☒	89	B.5 Compositional analyses
☒	102	C Nutritional impact of GM food
☒	102	D Other information

Part 2 Specific Data Requirements for Safety Assessment

The following information is provided to support an application for a new genetically modified food. The details presented are in accordance with Section 3.5.1. of the FSANZ Application Handbook as at 1 July 2019.

Table of Contents

Part 1 General Requirements (3.1.1) 2
A. Executive Summary 2
B. Applicant Details 3
C. Purpose of the Application 4
D. Justification for the Application 4
E. Information to Support the Application 5
F. Assessment Procedure 6
G. Confidential Commercial Information (CCI) 6
Release of Information 6
H. Other Confidential Information 6
I. Exclusive Capturable Commercial Benefit 6
Costs and Benefits 6
J. International and Other National Standards 10
K. Statutory Declaration – Australia 12
L. Checklists Provided With Application 13
General Requirements 13
Foods Produces Using Gene Technology 14
Part 2 Specific Data Requirements for Safety Assessment 15
Table of Contents 16
List of Tables 19
List of Figures 20
Abbreviations, Acronyms and Definitions 21
A. Technical Information on the Food Produced Using Gene Technology 23
A.1. Nature and Identity of the Genetically Modified Food 23
A.1(a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification. 23
A.1(b) The name, number or other identifier of each of the new lines or strains of GM organism from which the food is derived. 23
A.1(c) The name the food will be marketed under (if known). 24
A.2. History of use of the host and donor organisms 24
A.2(a) For the donor organism(s) from which the genetic elements are derived: 24
A.2(a)(i) Any known pathogenicity, toxicity or allergenicity of relevance to the food 24
A.2(a)(ii) History of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant) 29
A.2(b) A description of the host organism into which the genes were transferred: 29
A.2(b)(i) Its history of safe use for food 29
A.2(b)(ii) The part of the organism typically used as food 29
A.2(b)(iii) The types of products likely to include the food or food ingredient 29
A.2(b)(iv) Whether special processing is required to render food derived from the organism safe to eat 30
A.3. The nature of the genetic modification 30
A.3(a) A description of the method used to transform the host organism 30
Conclusion of the Development of event Z6 31
A.3(b) A description of the construct and the transformation vectors used 34
Potato Genes Targeted for Down Regulation using RNAi 39
Gene for Late Blight Protection 39
A.3(c) A full molecular characterisation of the genetic modification in the new organism 40
Structure of the pSIM1278 and pSIM1678 Inserts in Z6 40

Characterisation of the pSIM1278 and pSIM1678 Inserts in Z6 55
Absence of Vector Backbone DNA 59
Conclusion 60
A.3(c)(iv) A map depicting the organisation of the inserted genetic material at each insertion site 60
A.3(c)(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs) 60
A.3(d) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process) including which generations have been used. 61
A.3(e) Evidence of the stability of the genetic changes, including: 63
(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored 63
(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments 63
Stability of Event Z6 63
A.3(g) An analysis of the expressed RNA transcripts, where RNA interference has been used 68
Target Transcript Down Regulation in event Z6 68
PPO Activity in Event Z6 70
Conclusion of the Target Transcript Down Regulation of Event Z6 71
Conclusion of the Genetic Characterisation of Z6 71
B. Characterisation and Safety Assessment of New Substances 72
B.1. Characterisation and Safety Assessment of New Substances 72
B.1(a) a full description of the biochemical function and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions 72
VNT1 Expression in Event Z6 72
CONCLUSION 74
B.1(b) Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food. 75
Safety Assessment of the VNT1 Protein 75
B.1(c) information on whether any new protein has undergone any unexpected post-translational modification in the new host 76
B.1(d) where any ORFs have been identified (in subparagraph A.3(c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs 76
Identification of ORFs Associated with pSIM1278 and pSIM1678 77
Allergenicity Searches 77
Gene Sequences 78
Junction Sequences 78
Insert Sequences 78
Toxicity Searches 79
Gene Sequences 79
Junction Sequences 81
Insert Sequences 81
B.2. New Proteins 85
B.2 (a) and (b) Information on potential toxicity and allergenicity 85
VNT1 Protein Identity 85
B.3. Other (non-protein) new substances 86
B.3(a) the identity and biological function of the substance 86
B.3(b) whether the substance has previously been safely consumed in food 86
B.3(c) potential dietary exposure to the substance 86

B.3(d)(i) where RNA interference has been used: the role of any endogenous target gene and any changes to the food as a result of silencing that gene 86
B.3(d)(ii) where RNA interference has been used: the expression levels of the RNA transcript 87
B.3(d)(iii) where RNA interference has been used: the specificity of the RNA interference 87
B.5 Compositional analyses of the food produced using gene technology 88
Compositional Assessment of V11 and Z6 88
Conclusion of the Compositional Assessment of V11 and Z6 95
Low acrylamide potential and lowered reducing sugars 95
Free amino acids 95
Reducing Sugars 97
Summary of Reducing Sugars in V11 and Z6 98
Acrylamide Potential 98
Summary of Acrylamide Potential in V11 and Z6 100
C. Information related to the nutritional impact of the genetically-modified food 101
D. Other Information 101
Reports 102
References 103

List of Tables

Table 1: Current Applications and Approval Status for SPS-∅∅V11-6, and SPS-∅∅∅Z6-5 11
Table 2: Summary of Genes, Intended Traits, and Benefits in event Z6 23
Table 3. Genetic Elements of pSIM1278, from Left Border to Right Border 26
Table 4. Genetic Elements of pSIM1678, from Left Border to Right Border 28
Table 5. Synopsis of Simplot Biotech Potato Events 31
Table 6. Genetic Elements of the pSIM1278 and pSIM1678 Plasmid Backbone 37
Table 7. Summary of Down-Regulated Transcripts in Z6 Plants 68
Table 8. Overview of Analyses Using Bioinformatics 76
Table 9. Allergen Homology of pSIM1678 ORFs in Z6 78
Table 10. Summary of Toxin Homology for VNT1 80
Table 11. Toxin Search Results for pSIM1278 ORFs 82
Table 12. Toxin Search Results for pSIM1678 ORFs 83
Table 13. Proximates, Vitamins, and Minerals in V11 and Snowden 89
Table 14. Proximates, Vitamins, and Minerals in Z6 and Snowden 90
Table 15. Total Amino Acids in V11 and Snowden 92
Table 16. Total Amino Acids in Z6 and Snowden 93
Table 17. Glycoalkaloids in Tubers from V11 and Control Snowden 94
Table 18. Glycoalkaloids in Tubers of Z6 and Snowden 94
Table 19. Free Amino Acids in Tubers from V11 and Control Snowden 96
Table 20. Free Amino Acids in Tubers of Z6 and Snowden 96
Table 21. Sugars in Tubers from V11 and Control Snowden at Harvest and After Storage at 10 °C 97
Table 22. Sugars in Tubers of Z6 and Snowden at Harvest 98
Table 23. Acrylamide in Chips from V11 and Snowden at Harvest and After Storage at 10 °C 99
Table 24. Acrylamide in Chips from Z6 and Snowden at Harvest 100

List of Figures

Figure 1. The Development and Selection of Event V11 Transformed with pSIM1278 32
Figure 2. Development and Selection of Event Z6 Transformed with pSIM1678 33
Figure 3. Plasmid Map of pSIM1278 35
Figure 4.Plasmid Map of pSIM1678 36
Figure 5. pSIM1278 and pSIM1678 Copy Number in Z6 Using pSIM1278 Probes 41
Figure 6. Structure of the pSIM1278 Insert in Z6 with Digestion Patterns and Probe Binding Sites 43
Figure 7. Southern Hybridization of Genomic DNA with AGP Probe 44
Figure 8. Southern Hybridization of Genomic DNA with GBS Probe 45
Figure 9. Southern Hybridization of Genomic DNA with AGP Probe 46
Figure 10. Southern Hybridization of Genomic DNA with GBS Probe 47
Figure 11. Southern Hybridization of Genomic DNA with ASN Probe 48
Figure 12. Southern Hybridization of Genomic DNA with R1 Probe 49
Figure 13. Structure of the pSIM1678 Insert in Z6 with Digestion Patterns and Probe Binding Sites 51
Figure 14. Southern Hybridization of Genomic DNA with INV Probe 52
Figure 15. Southern Hybridization of Genomic DNA with VNT Probe 53
Figure 16. Southern Hybridization of Genomic DNA with INV Probe 54
Figure 17. Insert Structures in Z6 with Junctions Labeled 55
Figure 18. Summary of the Sequence Characterization of the pSIM1278 and pSIM1678 Inserts 57
Figure 19. Characterization of the pSIM1278 Insertion Site on Chromosome 4 58
Figure 20. Characterisation of the pSIM1678 Insertion Site on Chromosome 9 59
Figure 21. Illumina sequencing reads aligned to the binary vector backbone 60
Figure 22. Potato Plant and Tuber Propagation 62
Figure 23. Commercial Production of Potatoes 62
Figure 24. Vegetative Propagation of Potato Plants 63
Figure 25. Structure and Expected Digestion Pattern for Z6 Inserts from pSIM1278 and pSIM1678 64
Figure 26. EcoRV Digests: pSIM1278 and pSIM1678 Z6 Inserts are Stable during Vegetative Propagation . 66
Figure 27. Mfel/Xbal Digests: pSIM1278 and pSIM1678 Z6 Inserts are Stable during Vegetative Propagation 67
Figure 28. Target Transcript Down Regulation in Z6 Tubers 69
Figure 29. Reduced PPO Activity in Z6 Tubers 70
Figure 30. VNT1 was Undetected in Z6 by Western Blot 73
Figure 31. *Rpi-vnt1* Transcript Levels in Z6 Measured by RT-qPCR 74
Figure 32. Complete ORF Analysis Scheme 77
Figure 33. Alignment of VNT1 with Disease Resistance Protein, RGA2-like, from Apple 81
Figure 34. Amino Acid Sequence of VNT1 85

Abbreviations, Acronyms and Definitions[5]

Abbreviation	Definition
ADP	Adenosine diphosphate
AGP	Probe used to detect *Agp* promoter sequence
Agp	ADP-glucose pyrophosphorylase gene
A. tumefaciens	*Agrobacterium tumefaciens*
AP1	Adapter primer 1
AP2	Adapter primer 2
ASN	Probe used to detect *Asn1* sequence
ASN1	*Asn1* gene-derived probe used in DNA gel blot hybridization
Asn1	Asparagine synthetase-1 gene
ATP	Adenosine triphosphate
AUDPC	Area under disease progress curve
AUG	Start codon
Backbone DNA	DNA associated with construct backbone
bp	Base pair
CC	Coiled-coil domain
CFR	Code of Federal Regulations
CPC	Canadian Potato Council
cwt/A	Unit of measure equal to 100 lbs/acre or weight (lbs) of tubers harvested/acre divided by 100
DIGII	Molecular weight markers (125 bp – 23,130 bp)
DIGVII	Molecular weight markers (81 bp – 8,576 bp)
DNA insert	DNA sequence from pSIM1278 or pSIM1678 integrated into the potato genome
dNTP	Deoxy nucleotide triphosphate
dsRNA	Double-stranded RNA
ETS	Excellence Through Stewardship
FARRP	Food Allergy Resource Research Program and the University of Nebraska Lincoln
FDA	Food and Drug Administration
GBS, GBS1	Probe used to detect one region of the *Gbs* promoter sequence
GBS2	Probe used to detect second region of the *Gbs* promoter sequence
Gbss	Granule-bound starch synthase gene
IB	Internal band
ILSI	International Life Sciences Institute
Innate®	A branded biotechnology approach that uses plant genes to enhance desired traits
INV	Probe used to detect *VInv* sequence
IPM	Integrated pest management
ipt	Isopentenyltransferase gene—produces cytokinin hormones associated with plant growth and development
JB	Junction band
Kan^R	Kanamycin resistance gene provides a selectable marker for maintenance in bacteria
kb	Kilobase
LB	Left border

[5] NOTE: Abbreviations of units of measure and of physical and chemical quantities are used according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

LOD	Limit of detection
LOQ	Limit of quantification
LRR	Leucine-rich repeat domain
MOPS	3-(*N*-morpholino)propanesulfonic acid
N. benthamiana	*Nicotiana benthamiana*
NB	Nucleotide-binding
NB-VNT1	full-length VNT1 expressed and enriched from *N. benthamiana*
NBY	Nutrient broth-yeast extract
NBS-LRR	Nucleotide binding site/ leucine rich repeat
NGS	Next generation sequencing
OECD	Organisation for Economic Cooperation and Development
ORF	Open reading frame
P. infestans	*Phytophthora infestans*
pAgp	Promoter of the ADP glucose pyrophosphorylase gene
pBR322	Backbone element
PCR	Polymerase chain reaction
pGbss	Promoter of the granule-bound starch synthase gene
PhL	Phosphorylase-L gene
PPO	Polyphenol oxidase enzyme
Ppo5	Polyphenol oxidase-5 gene
qPCR	Quantitative PCR
pVnt1	*Rpi-vnt1* promoter
pVS1	Backbone element
R-protein	Resistance protein
R-gene	Resistance gene
R1	Southern blot probe used to detect the R1 cassette
R1	Water dikinase R1 gene
RB	Right border
Rpi-vnt1	Protection gene against *P. infestans*
RT-qPCR	Reverse transcription-qualitative polymerase chain reaction
S. phureja	*Solanum phureja*
S. tuberosum	*Solanum tuberosum*
S. venturii	*Solanum venturii*
SDS	Sodium dodecyl sulfate
Somaclonal variation	Genetic and/or phenotypic variation among propagated plants from a single parent arising from the callus phase of tissue culture
SPS-∅∅V11-6	OECD identifier for V11
SPS-∅∅∅Z6-5	OECD identifier for Z6
tVnt1	*Rpi-vnt1* terminator
Ubi7	Polyubiquitin 7 promoter
Ubi3	Polyubiquitin 3 terminator
USDA-APHIS	United States Department of Agriculture-Animal and Plant Health Inspection
VInv	Vacuolar invertase
VNT	Probe used to detect *Rpi-vnt1* sequence
VNT1	VNT1 protein
WT	Wild-type

A. Technical Information on the Food Produced Using Gene Technology

A.1. Nature and Identity of the Genetically Modified Food

A.1(a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification.

The event Z6 was developed by transforming the potato variety Snowden, with the *Agrobacterium* vectors pSIM1278 and pSIM1678. The event was developed to confer late blight protection, lower levels of free asparagine and reducing sugars, and reduced polyphenol oxidase, which contribute to reduced acrylamide potential and reduced black spot (Table 2).

The vector pSIM1278 contains two cassettes for the down-regulated expression of four specific potato enzymes (asparagine synthetase, polyphenol oxidase, water dikinase, and phosphorylase). The vector pSIM1678 also contains two cassettes, one for the expression of a wild potato gene to convey late blight protection and one for the down regulated expression of a specific potato enzyme vacuolar invertase.

Table 2: Summary of Genes, Intended Traits, and Benefits in event Z6

Construct	Gene Target	Mechanism	Intended Trait	Intended Benefit
pSIM1278	*Asn1*: asparagine synthetase-1[1]	RNAi down regulation	Reduces free asparagine	Contributes to low acrylamide potential[3]
	R1: water dikinase[1]	RNAi down regulation	Lowers reducing sugars	Contributes to low acrylamide potential[3]
	PhL: phosphorylase-L[1]	RNAi down regulation	Lowers reducing sugars	Contributes to low acrylamide potential[3]
	Ppo5: polyphenol oxidase-5[1]	RNAi down regulation	Reduces enzymatic darkening	Reduced black spot
pSIM1678	*VInv*: vacuolar invertase[2]	RNAi down regulation	Lowers reducing sugars	Contributes to low acrylamide potential[3]
	Rpi-vnt1: R-gene[2]	Protein expression	Confers protection against *P. infestans*	Late blight protection

[1] Previously evaluated by FSANZ in Application A1128 and A1139.
[2] Previously evaluated by FSANZ in Application A1139.
[3] Acrylamide is formed primarily from asparagine and reducing sugars heated at temperatures above 120 °C, as occurs during frying.

A.1(b) The name, number or other identifier of each of the new lines or strains of GM organism from which the food is derived.

In accordance with OECD '*Guidance for the Designation of a Unique Identifier for Transgenic Plants*', the OECD Unique Identification Code for the potato events are Snowden events SPS-∅∅∅Z6-5 and SPS-∅∅V11-6.

A.1(c) The name the food will be marketed under (if known).

The potato containing the Innate® technology will be marketed on the Canadian and United States fresh potato market as:

- Event Z6–Elevate

This potato will be marketed under a variety of labels as fresh cut, fries or crisps, depending on the licenced user of the event. Event V11–Invigorate, is not intended to be commercialised.

A.2. History of use of the host and donor organisms

A.2(a) For the donor organism(s) from which the genetic elements are derived:

A.2(a)(i) Any known pathogenicity, toxicity or allergenicity of relevance to the food

Donor DNA in the insert consists of both coding and non-coding genetic elements from two plasmids pSIM1278 and pSIM1678 as described in Table 3 and Table 4. All sequences from pSIM1278 are from *Solanum tuberosum* var. Ranger Russet (Potato) except for 2 elements derived from *S. verrucosum,* a diploid (2n=24) wild potato species from Mexico. Similarly, all sequences from pSIM1678 are from *Solanum tuberosum* var. Ranger Russet (Potato) with the addition of the native *Rpi-vnt1* promoter, gene and termination sequence first cloned from a South American wild *Solanum* species, *S. venturii* (Foster et al., 2009; Pel, 2010).

Details of the pathogenicity, toxicity or allergenicity of potato are described in the OECD Consensus Document on Compositional Considerations for New Varieties of Potatoes: Key Food and Feed Nutrients, Anti-nutrients and Toxicants (OECD, 2002).

Potatoes are not known to cause disease in humans or animals and have a long history of safe use as a food. Several features of this commodity relate to toxicity and allergenicity and are briefly discussed below.

Bioinformatic analyses confirm that VNT1 lacks significant homology to known toxins and allergens. Additionally, bioinformatic analyses demonstrate that proteins highly similar to VNT1 are present in the human diet, supporting a history of safe use. R-proteins like VNT1 do not confer pest protection by directly targeting the pest or by acting as toxins. Instead, VNT1 activates the plant's native, immune pathway resulting in a hypersensitive response. These factors contribute to the low hazard potential of VNT1.

All potatoes contain natural toxins called glycoalkaloids, the most prevalent of which are solanine and chaconine. Solanine is also found in other plants in the family Solanaceae, which includes plants such as the edible crops eggplant and tomato.

Potatoes are not among the 'Big Eight' group of foods that account for ~90% of all food allergies in the U.S. (FARRP, 2014). There are a few reports of allergies to cooked potato in children (DeSwert et al., 2002, 2007). However, most children with potato allergy develop tolerance at an average age of four years (De Swert et al., 2007). Patatin (Sol t 1) has been identified as the primary allergen involved in this reaction (Astwood et al., 2000). Patatin is a storage glycoprotein that displays lipase activity and makes up about 40% of the soluble protein in tubers (Mignery et al., 1988). There is no mechanistic reason to suggest that the level of patatin would be changed in Z6. Because potato protein naturally contains a relatively large proportion of patatin, any unexpected change in patatin levels would be unlikely to affect allergenicity enough to alter consumption patterns for people allergic to potatoes.

No sequences associated with either glycoalkaloids or patatin proteins were used in creating the potato events in this application.

ORDER: Solanales

FAMILY: Solanaceae

GENUS: *Solanum*

SPECIES: *S. tuberosum*, *S. verrucosum* Schltdl and *S. venturii*

COMMON NAME: Potato, Wild Potato

Table 3. Genetic Elements of pSIM1278, from Left Border to Right Border

Genetic Element	***Origin***	**Accession Number**	**Position (pSIM1278)**	**Size (bp)**	**Intended Function**
1. Left Border (LB) site[1]	Synthetic	AY566555[2]	1–25	25	Secondary cleavage site releases ssDNA insert from pSIM1278 (van Haaren et al., 1989)
2. LB region sequence	*S. tuberosum* var. Ranger Russet	AY566555[2]	26–187	162	Buffer for truncations during insertion
3. Intervening Sequence	*S. tuberosum*	AF393847	188 –193	6	Sequence used for DNA cloning
4. ADP glucose pyrophosphorylase gene promoter (*pAgp*), 1st copy	*S. tuberosum* var. Ranger Russet	HM363752	194-2,453	2260	Drives expression of *Asn1* and *Ppo5* inverted repeats, especially in tubers
5. *Asn1* gene fragment (1st copy, antisense orientation)	*S. tuberosum* var. Ranger Russet	HM363759	2,454-2,858	405	Generates dsRNA that down regulates asparagine synthetase to impair asparagine formation (Chawla et al., 2012[3])
6. Fragment of the 3'-untranslated region of the polyphenol oxidase-5 gene (*Ppo5*) (1st copy, antisense orientation)	*S. verrucosum*	HM363754	2,859-3,002	144	Generates dsRNA that triggers the down regulation of PPO to reduce black spot development
7. Intervening Sequence	*S. tuberosum*	DQ478950	3,003-3,008	6	Sequence used for DNA cloning
8. Spacer-1	*S. tuberosum* var. Ranger Russet	HM363753	3,009-3,165	157	Sequence between the 1st inverted repeat; transcript forms loop in dsRNA
9. Fragment of the 3'-untranslated region of the polyphenol oxidase-5 gene (*Ppo5*) (2nd copy, sense orientation)	*S. verrucosum*	HM363754	3,166-3,309	144	Generates dsRNA that triggers the down regulation of PPO to reduce black spot development
10. *Asn1* gene fragment (2nd copy, sense orientation)	*S. tuberosum* var. Ranger Russet	HM363759	3,310-3,715	406	Generates dsRNA that down regulates asparagine synthetase to impair asparagine formation (Chawla et al., 2012[3])
11. Intervening Sequence	*S. tuberosum*	X73477	3,716-3,721	6	Sequence used for DNA cloning
12. Granule-bound starch synthase gene promoter (*pGbss*) (opposite direction from 1st copy of *pAgp*)	*S. tuberosum* var. Ranger Russet	HM363755	3,722-4,407	686	Drives expression of *Asn1* and *Ppo5* inverted repeats, especially in tubers
13. Intervening Sequence	*S. tuberosum*	X95996 / AF393847	4,408-4,423	16	Sequence used for DNA cloning
14. *pAgp*, 2nd copy	*S. tuberosum* var. Ranger Russet	HM363752	4,424-6,683	2260	Drives expression of PhL and R1 inverted repeats , especially in tubers

Genetic Element	*Origin*	Accession Number	Position (pSIM1278)	Size (bp)	Intended Function
15. Fragment of the region extending from the 5'-untranslated region into the promoter of the potato phosphorylase-L (*PhL*) gene (1st copy, antisense orientation)	*S. tuberosum* var. Ranger Russet	HM363758	6,684-7,192	509	Generates dsRNA that triggers the degradation of *PhL* transcripts to limit the formation of reducing sugars
16. Fragment of promoter for the potato *R1* gene (1st copy, antisense orientation)	*S. tuberosum* var. Ranger Russet	HM363757	7,193-7,724	532	Generates dsRNA that triggers the degradation of *R1* transcripts to limit the formation of reducing sugars
17. Intervening Sequence	*S. tuberosum*	DQ478950	7,725-7,730	6	Sequence used for DNA cloning
18. Spacer-2	*S. tuberosum* var. Ranger Russet	U26831[4]	7,731-7,988	258	Sequence between the 2nd inverted repeat; transcript forms loop in dsRNA
19. Fragment of promoter for the potato *R1* gene (2nd copy, sense orientation)	*S. tuberosum* var. Ranger Russet	HM363757	7,989-8,520	532	Generates dsRNA that triggers the degradation of *R1* transcripts to limit the formation of reducing sugars
20. Fragment of the region extending from the 5'-untranslated region into the promoter of the potato phosphorylase-L (*PhL*) gene (2nd copy, sense orientation)	*S. tuberosum* var. Ranger Russet	HM363758	8,521-9,029	509	Generates dsRNA that triggers the degradation of *PhL* transcript to limit the formation of reducing sugars
21. *pGbss* (2nd copy, opposite direction from 2nd copy of *pAgp*)	*S. tuberosum* var. Ranger Russet	X83220[5]	9,030-9,953	924	Drives expression of PhL and R1 repeats, especially in tubers
22. Intervening Sequence	*S. tuberosum*	AF143202	9,954 – 9,962	9	Sequence used for DNA cloning
23. Right Border (RB) region sequence	*S. tuberosum* var. Ranger Russet	AY566555[2]	9,963 – 10,123	161	Buffer for truncations during insertion
24. RB sequence[1]	Synthetic	AY566555[2]	10,124 – 10,148	25	Primary cleavage site releases ssDNA insert from pSIM1278 (van Haaren et al., 1989)

[1]The LB and RB sequences (25-bp each) were synthetically designed to be similar to and function like T-DNA borders from *Agrobacterium tumefaciens*.
[2]GenBank Accession AY566555 was revised to clarify the sources of DNA for the border regions.
[3]ASN1 described as genetic elements 5 and 10 is referred to as StAst1 in Chawla et al., 2012.
[4]GenBank Accession HM363756 is replaced with a citation to GenBank Accession U26831 to include four 3' end nucleotides present in the pGbss DNA element of the pSIM1278 construct.
[5]GenBank Accession HM363755 is replaced with a citation to GenBank Accession X83220 to include the full *pGbss* (2nd copy) DNA insert sequence present in the pSIM1278 construct.

Table 4. Genetic Elements of pSIM1678, from Left Border to Right Border

Genetic Element	*Origin*	Accession Number	Position (pSIM1678)	Size (bp)	Intended Function
1. Left Border (LB) site[1]	Synthetic	AY566555[3]	1–25	25	Secondary cleavage site releases ssDNA insert from pSIM1678 (van Haaren et al., 1989)
2. LB region	*S. tuberosum* var. Ranger Russet	AY566555[3]	26-187	162	Buffer for truncations during insertion
3. Intervening Sequence	*S. tuberosum*	AF393847	188–193	6	Sequence used for DNA cloning
4. Native *Rpi-vnt1* gene promoter	*S. venturii*	FJ423044	194 -902	709	Drives expression of the *Rpi-vnt1* gene
5. *Rpi-vnt1* gene coding sequence	*S. venturii*	FJ423044	903 – 3,578	2676	Expresses the VNT1 protein for late blight protection
6. Native *Rpi-vnt1* gene terminator	*S. venturii*	FJ423044	3,579 – 4,503	925	Terminates transcription of *Rpi-vnt1*
7. Intervening Sequence	*S. tuberosum*	HM363755	4,504 - 4,510	7	Sequence used for DNA cloning
8. ADP glucose pyrophosphorylase gene promoter (*pAgp*)	*S. tuberosum* var. Ranger Russet	HM363752	4,511 - 6,770	2260	Drives expression of the *VInv* inverted repeat, especially in tubers
9. Intervening Sequence	*S. tuberosum* var. Ranger Russet	DQ206630	6,771 - 6,776	6	Sequence used for DNA cloning
10. *VInv* gene fragment (sense orientation)	*S. tuberosum* var. Ranger Russet	DQ478950	6,777 – 7,274	498	Generates dsRNA to down regulate *VInv* transcripts
11. *VInv* gene fragment (sense orientation)	*S. tuberosum* var. Ranger Russet	DQ478950	7,275 – 7,455	181	Sequence between the inverted repeat; transcript forms loop in dsRNA
12. Intervening Sequence	*S. tuberosum* var. Ranger Russet	X73477	7,456 - 7,461	6	Sequence used for DNA cloning
13. *VInv* gene fragment (anti-sense orientation)	*S. tuberosum* var. Ranger Russet	DQ478950	7,462 - 7,959	498	Generates dsRNA to down regulate *VInv* transcripts
14. Intervening Sequence	*S. tuberosum* var. Ranger Russet	X95996	7,960 - 7,971	12	Sequence used for DNA cloning
15. Granule-bound starch synthase gene promoter (*pGbss*) (opposite direction from *pAgp*)	*S. tuberosum* var. Ranger Russet	X83220[2]	7,972 - 8,894	923	Drives expression of the *VInv* inverted repeat, especially in tubers
16. Intervening Sequence	*S. tuberosum*	AF143202	8,895 – 8,903	9	Sequence used for DNA cloning
17. Right Border (RB) region	*S. tuberosum* var. Ranger Russet	AY566555[3]	8,904– 9,064	161	Buffer for truncations during insertion
18. RB sequence[1]	Synthetic	AY566555[3]	9,065 – 9,089	25	Primary cleavage releases ssDNA insert from pSIM1678 (van Haaren et al., 1989)

[1]The LB and RB sequences (25-bp each) were synthetically designed to be similar to and function like T-DNA borders from *Agrobacterium tumefaciens*.

[2]GenBank Accession HM363755 is replaced with a citation to GenBank Accession X83220 to include the full *pGbss* (2nd copy) DNA insert sequence present in the pSIM1278 construct.

[3]GenBank Accession AY566555 was revised to clarify the sources of DNA for the Border regions

A.2(a)(ii) History of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant)

The potato is the world's fourth largest food crop, following rice, wheat, and maize. It has a long history in the diets of humans across the entire world.

The Inca Indians in Peru were the first to cultivate potatoes around 8,000 BC to 5,000 BC.

In 1536 Spanish Conquistadors conquered Peru, discovered the flavours of the potato, and carried them to Europe. Before the end of the sixteenth century, families of Basque sailors began to cultivate potatoes along the Biscay coast of northern Spain. Sir Walter Raleigh introduced potatoes to Ireland in 1589 on the 40,000 acres of land near Cork. It took four decades for the potato to spread to the rest of Europe.

Potatoes arrived in the Colonies in 1621 and the first permanent potato patches in North America were established in 1719. From there, the crop spread across the United States.

Potatoes were introduced into Australia and New Zealand with the early European settlers in the late 18th century. In 1797, Governor Hunter reported that 11 acres (4.5 (ha)) were under potato crop in the Parramatta district west of Sydney. A decade later, this area had increased to 301 acres (122 ha); and nearly a century later in 1906, 119,000 acres (48,000 ha) of potatoes were under crop in Australia.

Today, potato production occurs around Australia with the exception of the far northern areas where temperatures exceed the optimal growing conditions for this cool-season crop. All states grow significant quantities of potatoes with predominant production in the cooler states of South Australia, Tasmania and Victoria.

Sources: International Year of the Potato (FAO, 2008); Feature Article: Potatoes – The world's favourite vegetable (Year Book Australia 2008, Australian Bureau of Statistics, 2008)

A.2(b) A description of the host organism into which the genes were transferred:

A.2(b)(i) Its history of safe use for food

See Section A.2(a) (ii).

A.2(b)(ii) The part of the organism typically used as food

Potato tubers are the only part consumed as food.

A.2(b)(iii) The types of products likely to include the food or food ingredient

In Australia and New Zealand, consumer research has confirmed that all major uses of potato involve some level of cooked preparation. The versatility of potato has led to a range of uses, with the majority being centred around dinner, and to lesser degree lunch time occasions. Seasonal influences have little bearing on the main uses of potato, reflecting patterns of consistent consumption year-round. Within the Australian and New Zealand diet, potatoes are prepared and consumed in the following forms:

- Boiled, Microwaved, Steamed
- Deep fried
- Mashed
- Roasted
- Baked/Grilled

- Salad – cooked
- Soup/Sauce
- Stir fry
- Juiced
- Sandwich/burger/wrap
- Snacks – potato chips / straws.

A.2(b)(iv) Whether special processing is required to render food derived from the organism safe to eat

Potato tubers for direct consumption should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins (OECD, 2002).

Potatoes are prepared and packaged fresh as well as processed for fries, chips and flakes.

Potatoes are prepared in many ways: skin-on or peeled, whole or cut up, with seasonings or without. The only requirement involves cooking to swell the starch granules. Most potato dishes are served hot, but some are first cooked, then served cold, notably potato salad and potato chips/crisps.

Other uses include:

- Used to brew alcoholic beverages such as vodka, potcheen, or akvavit
- Feed for domestic animals
- Potato starch is used in the food industry as, for example, thickeners and binders of soups and sauces, in the textile industry, as adhesives, and for the manufacturing of papers and boards
- Potato skins, along with honey, are a folk remedy for burns in India. Burn centres in India have experimented with the use of the thin outer skin layer to protect burns while healing.

A.3. The nature of the genetic modification

A.3(a) A description of the method used to transform the host organism

The event Z6 was developed by first transforming variety Snowden with the plasmid pSIM1278 to reduce expression of asparagine synthetase, polyphenol oxidase, and the starch-associated enzymes, water dikinase and phosphorylase L (event V11).

In order to reduce expression of the vacuolar invertase transcripts (*VInv*) and add the late blight protection gene (*Rpi-vnt1*), a second transformation was carried out with plasmid pSIM1678. These plasmids are described in detail in Table 3 and Table 4 and Section A3(b) and have been assessed previously by FSANZ in applications A1128 and A1139. A synopsis of events V11 and Z6 is provided in Table 5.

Table 5. Synopsis of Simplot Biotech Potato Events

Product category	Plasmids Used for Transformation	Variety
		Snowden
Gen1	pSIM1278	V11
Gen2	Gen1 + pSIM1678	Z6*

*Z6 was developed from V11, which was not commercialised.

The pSIM1278 and pSIM1678 transformation protocols are described in

Figure 1 and

Figure 2.

Somaclonal variation occurs when genetically dissimilar individuals are derived from vegetative propagation. *S. tuberosum* varieties are prone to somaclonal variation during the callus stage that occurs during transformation and may exhibit a degree of heterogeneity (OECD, 1997). Several steps were taken during event selection to mitigate somaclonal variation. These steps were:

1. A number of transformed plants were produced;
2. A late blight assay was used to screen for plants expressing the VNT1 protein;
3. Plants exhibiting partial protection against late blight were discarded;
4. Asymptomatic plants were selected and advanced for field testing; and
5. Any plants with off-types were removed.

The selection process was conducted by studying the growth characteristics of the transformed events compared to controls. Field trials evaluating phenotypic and agronomic characteristics did not identify any somaclonal variation in event Z6.

<u>Conclusion of the Development of event Z6</u>

Event Z6 was developed by transforming the potato variety Snowden with pSIM1278 and then retransforming with pSIM1678. Transformation introduced DNA sequences (*Asn1*, *R1*, *Ppo5*, *PhL*, and *VInv*) intended to down regulate asparagine synthetase, polyphenol oxidase, water dikinase, phosphorylase, and vacuolar invertase through the mechanism of RNAi. In addition, the late blight resistance gene, *Rpi-vnt1,* under the control of its native promoter and terminator was introduced.

All genetic elements between the LB and RB of the T-DNA in pSIM1278 and pSIM1678 were derived from the genomes of cultivated and wild solanum species. Several steps were taken throughout the transformation and selection process to discard any plants showing somaclonal variation or the presence of backbone sequence.

Potato events containing the T-DNA of both pSIM1278 and pSIM1678 have been assessed and approved by FSANZ in a previous submission A1139.

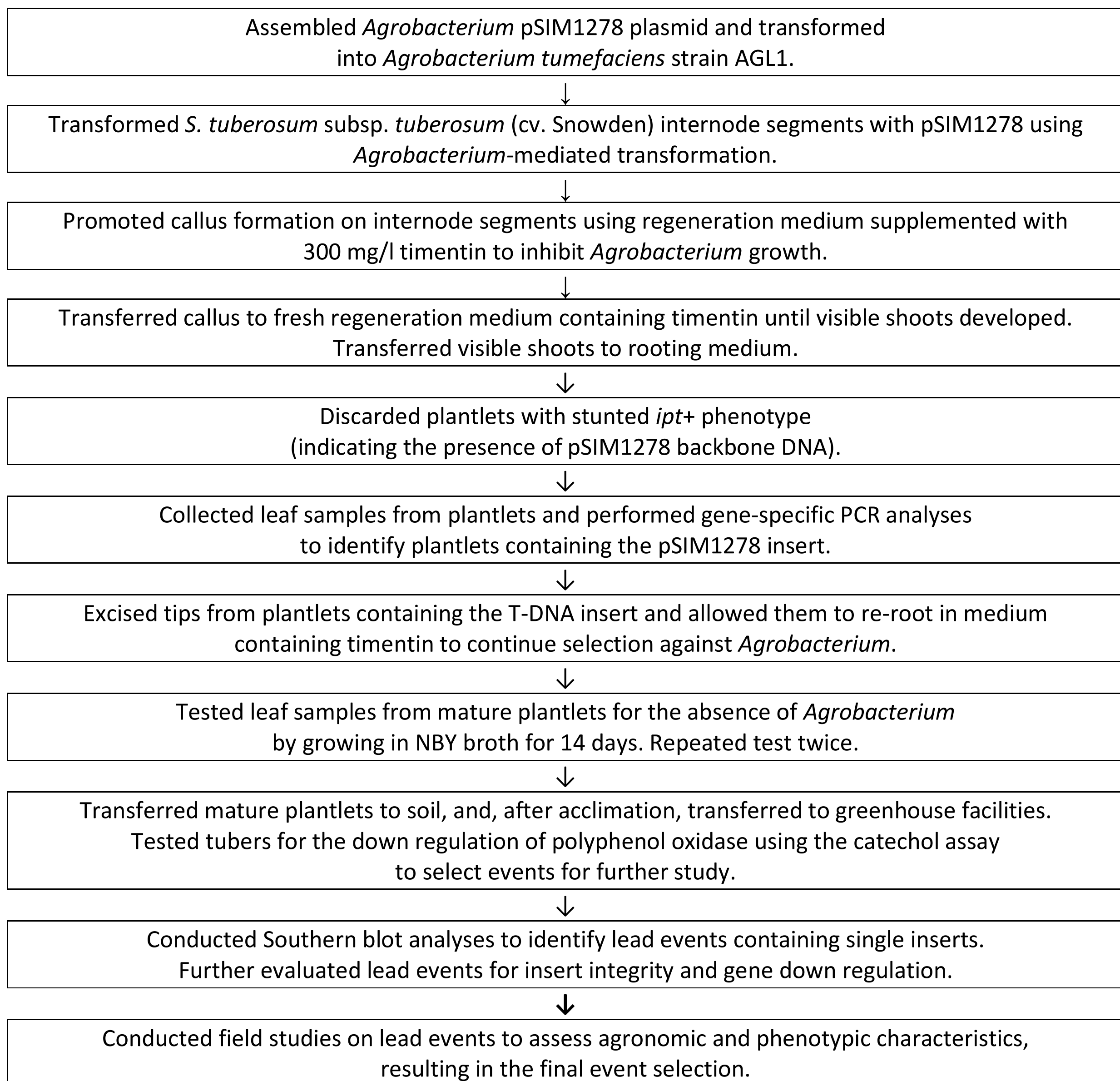


Figure 1. The Development and Selection of Event V11 Transformed with pSIM1278

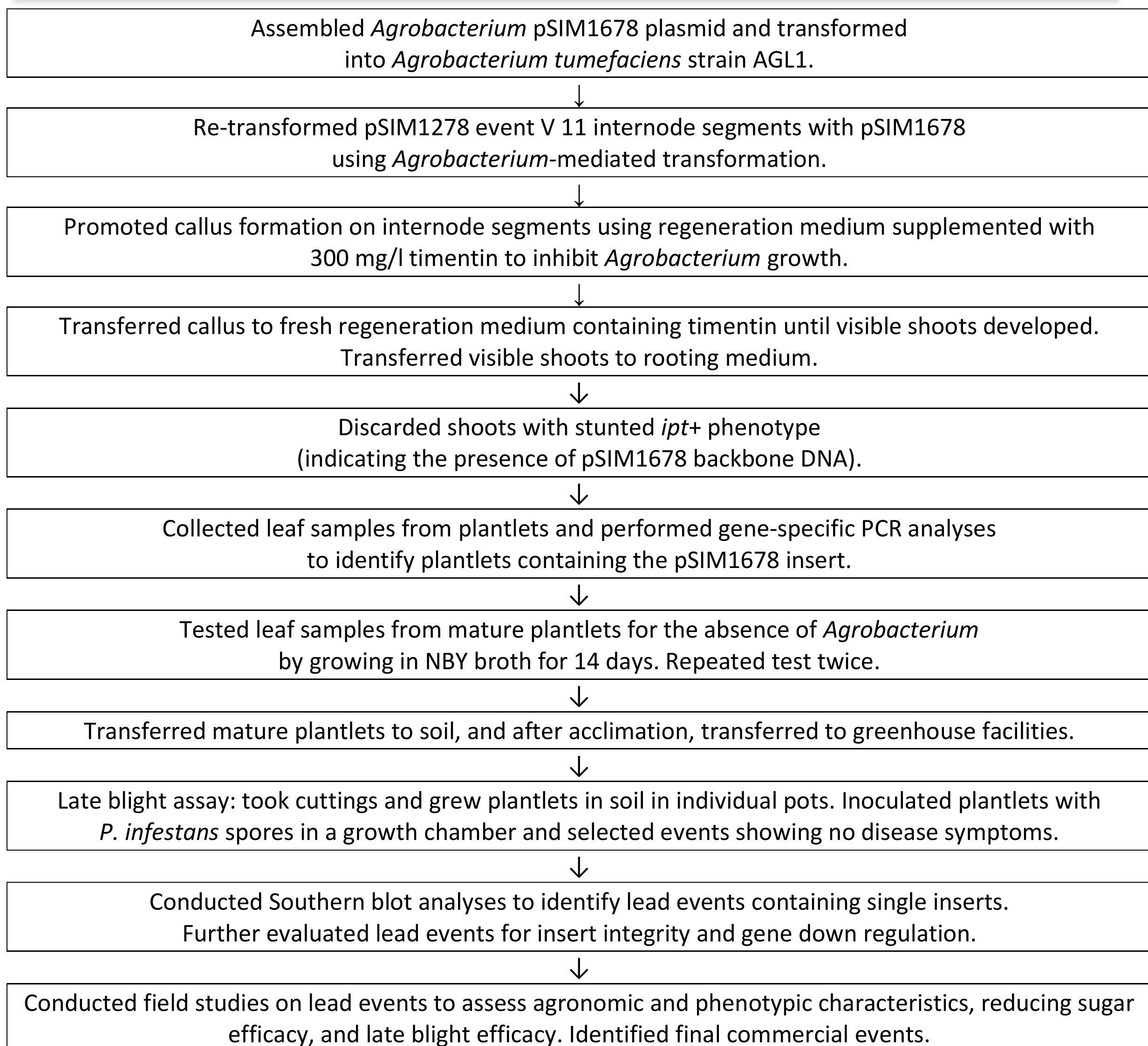


Figure 2. Development and Selection of Event Z6 Transformed with pSIM1678

A.3(b) A description of the construct and the transformation vectors used

Event Z6 was developed by transforming the potato variety Snowden with pSIM1278 followed by transformation with pSIM1678. All genetic elements between the left border (LB) and right border (RB) of the T-DNA in pSIM1278 and pSIM1678, were derived from the genomes of cultivated and wild potato species. The resulting varieties possess late blight protection and exhibit a reduction in black spot, reducing sugars, and acrylamide potential.

The pSIM1278 and pSIM1678 plasmids were constructed using the same parental plasmid and therefore share identical backbone sequences (Table 6). The backbone contains two well-characterised bacterial origins of replication:

- pVS1 (pVS1 Sta and Rep), which enables maintenance of the plasmid in *Agrobacterium* and
- pBR322 (pBR322 bom and ori), which enables maintenance of the plasmid in *Escherichia coli*.

Additional backbone elements include:

- The *Agrobacterium* DNA overdrive sequence enhances cleavage at the RB;
- The kanamycin resistance gene (Kan^R) functions as a selectable marker for maintenance in bacteria; and
- The *Agrobacterium* isopentenyl transferase (*ipt*) gene flanked by the Ranger Russet potato polyubiquitin (*Ubi7*) promoter and the Ranger Russet potato polyubiquitin (*Ubi3*) terminator (Garbarino and Belknap, 1994).

The backbone contains a cassette comprising the *Agrobacterium* isopentenyl transferase (*ipt*) gene flanked by the Ranger Russet potato polyubiquitin (*Ubi7*) promoter and the Ranger Russet potato polyubiquitin (*Ubi3*) terminator (Garbarino and Belknap, 1994). Although *Agrobacterium* is effective in cleaving at the RB aided by the overdrive sequence, cleavage at the LB is often less precise (Gelvin, 2003), resulting of the transfer of backbone sequence in addition to the T-DNA.

Overexpression of *ipt* was used as a phenotype screen to select against the integration of plasmid backbone DNA (Table 6). When present in transformed plants, overexpression of *ipt* results in the overproduction of the plant hormone, cytokinin. This causes plants with stunted phenotypes, abnormal leaves and the inability to form roots. Only plantlets that were phenotypically indistinguishable from non-transformed controls were selected and allowed to develop. This screening ensured that transformed plants lacked the functionally active backbone *ipt* marker gene (Richael et al., 2008).

Maps of pSIM1278 and pSIM1678, are provided in Figure 3 and Figure 4, respectively, with corresponding descriptions of the genetic elements in the T-DNA provided in Table 3 and Table 4.

The plasmid, pSIM1278, is a 19.6 kb binary transformation vector used in the first transformation of potato variety Snowden.

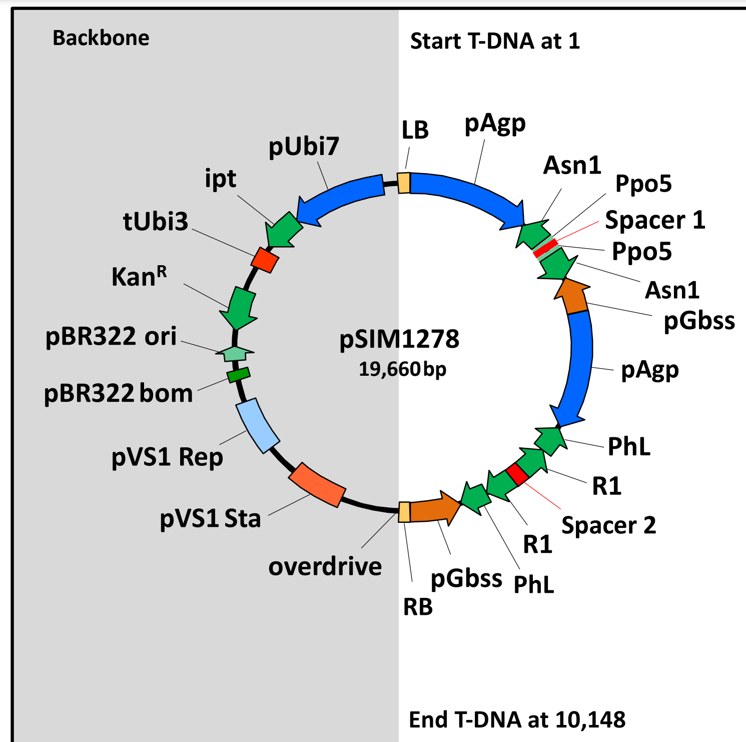


Figure 3. Plasmid Map of pSIM1278

The backbone region (grey background) starts at position 10,149 bp and ends at 19,660 bp. The backbone consists of bacterial and potato DNA and is intended only to support maintenance of the DNA insert prior to plant transformation. The T-DNA insert region (white background) is from 1 bp to 10,148 bp.

pSIM1278 T-DNA contains two down-regulation cassettes (Figure 3):

- The first (elements 4 to 12; Table 3) is designed to down regulate asparagine synthetase and polyphenol oxidase in the transformed potato variety. The inverted repeat is comprised of asparagine synthetase 1 (*Asn1*) and polyphenol oxidase 5 (*Ppo5*) fragments, separated by a spacer element (Spacer-1) and arranged between two convergent potato promoters: the *Agp* promoter of the ADP glucose pyrophosphorylase gene (*Agp*), and the *Gbss* promoter of the granule-bound starch synthase gene (*Gbss*). Both promoters are primarily active in tubers
- The second (elements 14 to 21; Table 3) is designed to down regulate phosphorylase L and water dikinase in the transformed potato variety. The inverted repeat is comprised of phosphorylase L (*PhL*) and water dikinase (*R1*) fragments, separated by a spacer element (Spacer-2) and arranged between the two convergent potato promoters, *Agp* and *Gbss*.

The plasmid, pSIM1678, is an 18.6 kb binary transformation vector. A plasmid map for pSIM1678 is provided in Figure 4. As mentioned above, the plasmids were constructed using the same parental plasmid and therefore share identical backbone sequences (Table 6).

pSIM1678 T-DNA contains the *Rpi-vnt1* cassette and a down-regulation cassette (Figure 4):

- The *Rpi-vnt1* cassette (elements 4 to 6; Table 4) contains the 2,676 bp *Rpi-vnt1* gene. The gene is expressed under the native promoter (*pVnt1*) and terminator (*tVnt1*) and

- The VInv down-regulation cassette, (elements 8 to 15; Table 4) is designed to down regulate vacuolar invertase (VINV) in the transformed potato variety. The inverted repeat is comprised of *VInv* fragments, separated by a spacer element (also a *VInv* fragment) and arranged between the two convergent potato promoters, *Agp* and *Gbss*.

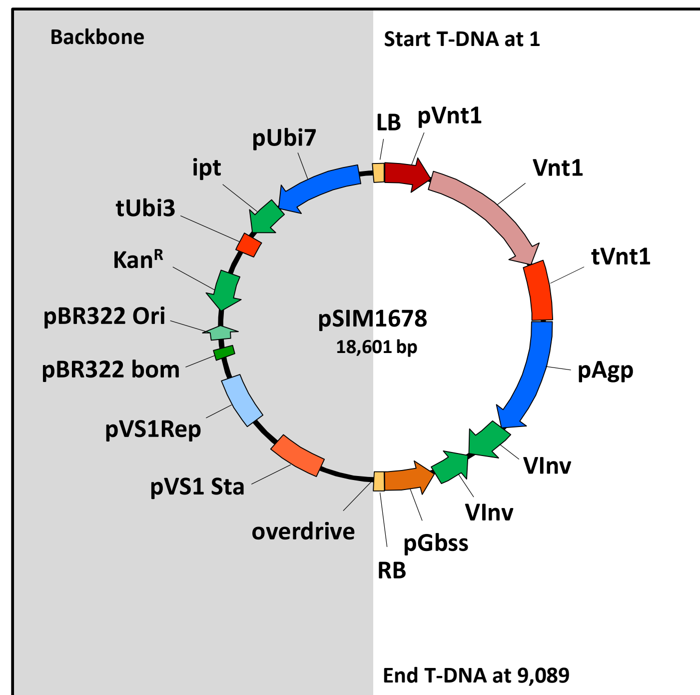


Figure 4.Plasmid Map of pSIM1678

The backbone region (grey background) starts at position 9,090 bp and ends at 18,601 bp. The backbone DNA is the same between plasmids pSIM1278 and pSIM1678. The backbone consists of bacterial and potato DNA and is intended only to support maintenance of the DNA insert prior to plant transformation. The T-DNA insert region (white background) is from 1 bp to 9,089 bp.

Table 6. Genetic Elements of the pSIM1278 and pSIM1678 Plasmid Backbone

Genetic Element	Origin	Accession Number[1]	Position for pSIM1278[1]	Size (bp)	Function
1. Intervening sequence	Synthetic DNA		10,149-10,154	6	Sequence used for cloning
2. Overdrive	*Agrobacterium tumefaciens* Ti-plasmid	NC_002377	10,155-10,184	30	Enhances cleavage at *A. tumefaciens* Right Border site [1]
3. Intervening sequence	*Pseudomonas fluorescens* pVS1	AJ537514	10,185-11,266	1,082	pVS1 backbone
4. pVS1 partitioning protein StaA (PVS1 Sta)	*P. fluorescens* pVS1	AJ537514	11,267-12,267	1,001	pVS1 stability
5. Intervening sequence	*P. fluorescens* pVS1	AJ537514	12,268-12,860	593	pVS1 backbone
6. pVS1 replicon (pVS1Rep)	*P. fluorescens* pVS1	AJ537514	12,861-13,861	1,001	pVS1 replication region in *Agrobacterium*
7. Intervening sequence	*P. fluorescens* pVS1	AJ537514	13,862-14,099	238	pVS1 backbone
8. Intervening sequence	pBR322	AF234297	14,100-14,270	171	pCambia1301 backbone
9. pBR322 bom	pBR322	AF234297	14,271-14,531	261	pBR322 region for replication in *E. coli*
10. Intervening sequence	pBR322	AF234297	14,532-14,670	139	pCambia1301 backbone
11. Origin of replication for pBR322 (pBR322 ori)	pBR322	AF234297	14,671-14,951	281	Bacterial origin of replication
12. Intervening sequence	pBR322	AF234297	14,952-15,241	290	pCambia1301 backbone
13. Aminoglycoside phosphotransferase gene	pCambia1301	AF234297	15,242-16,036	795	Kanamycin resistance gene
14. Intervening sequence	Vector DNA	FJ362602	16,037-16,231	195	pCambia1301 vector backbone
15. Terminator of the ubiquitin-3 gene (*tUbi3*)	*S. tuberosum*	GP755544	16,232-16,586	355	Terminator for *ipt* gene transcription (Garbarino and Belknap, 1994)
16. Intervening sequence	*A. tumefaciens* Ti-plasmid	NC_002377	16,587-16,937	351	Sequence used for DNA cloning
17. Isopentenyl transferase (*ipt*) gene	*A. tumefaciens* Ti-plasmid	NC_002377	16,938-17,660	723	Condensation of AMP and isopentenyl-pyrophosphate to form isopentenyl-AMP, a cytokinin in the plant. Results in abnormal growth phenotypes in plant (Smigocki and Owens, 1988)

Genetic Element	Origin	Accession Number[1]	Position for pSIM1278[1]	Size (bp)	Function
18. Intervening sequence	Synthetic DNA		17,661-17,672	12	Sequence used for DNA cloning
19. Polyubiquitin promoter (*pUbi7*)	*S. tuberosum* var. Ranger Russet	U26831	17,673-19,410	1,738	Promoter to drive expression of the *ipt* backbone marker gene (Garbarino et al., 1995)
20. Intervening sequence	Vector DNA	U10460	19,411-19,660	250	pZP200 vector backbone[1]

[1] Position numbers for pSIM1678 are different, but the elements are the same

Potato Genes Targeted for Down Regulation using RNAi

Transcription of the inverted repeats leads to down regulation of endogenous target genes through production of dsRNA and the plant's RNAi pathway. The inverted repeats are derived from the DNA sequences of five target potato genes (*Asn1*, *Ppo5*, *PhL*, *R1,* and *VInv*; Table 2).

The T-DNA in each plasmid contains down-regulation cassettes that result in the production of small interfering RNA (siRNA) in event Z6 using the plant's RNAi pathway. As described above, each down-regulation cassette is comprised of DNA sequence arranged as an inverted repeat.

Due to the nature of the inverted repeat sequences, their transcripts form dsRNA through complementary binding. The dsRNA act as a precursor for the plant's own RNAi post-transcriptional regulatory pathway. A cellular RNase III enzyme, Dicer, recognises and processes the precursor dsRNA into 21-24 bp duplexes termed siRNA. The siRNA bind with cellular proteins forming RNA Induced Silencing Complexes (RISC). The RISC selectively degrades one of the siRNA strands, referred to as the passenger strand. The remaining strand, referred to as the guide strand, targets the complementary sequence in an mRNA molecule. Once the guide strand pairs with an mRNA in a RISC complex, the mRNA molecule is cleaved and degraded, preventing protein translation (Chau and Lee, 2007; Fire et al., 1998).

Gene for Late Blight Protection

The *Rpi-vnt1* gene found in *S. venturii* and *S. phureja* confers protection against the oomycete that causes late blight disease in potatoes, *P. infestans*. Late blight protection was achieved in event Z6 by the addition of the *Rpi-vnt1* gene with its native promoter and termination sequence. The *Rpi-vnt1* gene was cloned from a wild *Solanum* species, *S. venturii,* and is identical to the *Rpi-phu1* gene found in *S. phureja* (Śliwka et al., 2013).

A.3(c) A full molecular characterisation of the genetic modification in the new organism

This Section provides information that addresses the requirements for Part A.3(c) A full molecular characterisation of the genetic modification in the new organism, including:

(i) Identification of all transferred genetic material and whether it has undergone any rearrangements;

(ii) A determination of the number of insertion sites, and the number of copies at each insertion site;

(iii) Full DNA sequence of each insertion site, including junction regions with the host DNA;

(iv) A map depicting the organisation of the inserted genetic material at each insertion site; and

(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs).

Further information is provided in:

- [unpublished report]
- [unpublished report]
- [unpublished report]

Structure of the pSIM1278 and pSIM1678 Inserts in Z6

A combination of Southern blot and DNA sequencing analysis was used to show that Z6 contains single, nearly full-length inserts from both pSIM1278 and pSIM1678.

Southern analysis was used to determine that the pSIM1278 insert in Z6 consists of a single, nearly full-length copy of the T-DNA (Figure 5). The junctions between the pSIM1278 insert and the plant genome were determined using DNA sequencing. A series of additional Southern blots, shown below, were used to confirm the structure of the pSIM1278 insert. AGP, ASN, GBS, and R1 probes were hybridized to genomic DNA following digestion with EcoRV, HindIII, and a double digest with EcoRI and ScaI. The restriction sites, digestion products and sizes, and probe binding sites are shown in Figure 6. The Snowden (WT) sample was analysed with and without spiked pSIM1278 (p1278) or pSIM1678 plasmid (p1678). The spiked plasmid was at a concentration of a single copy per genome equivalent, prior to digestion. The spiked plasmids serve as positive controls for sensitivity of the probes and as size markers for bands internal to the pSIM1278 or pSIM1678 insert and T-DNA.

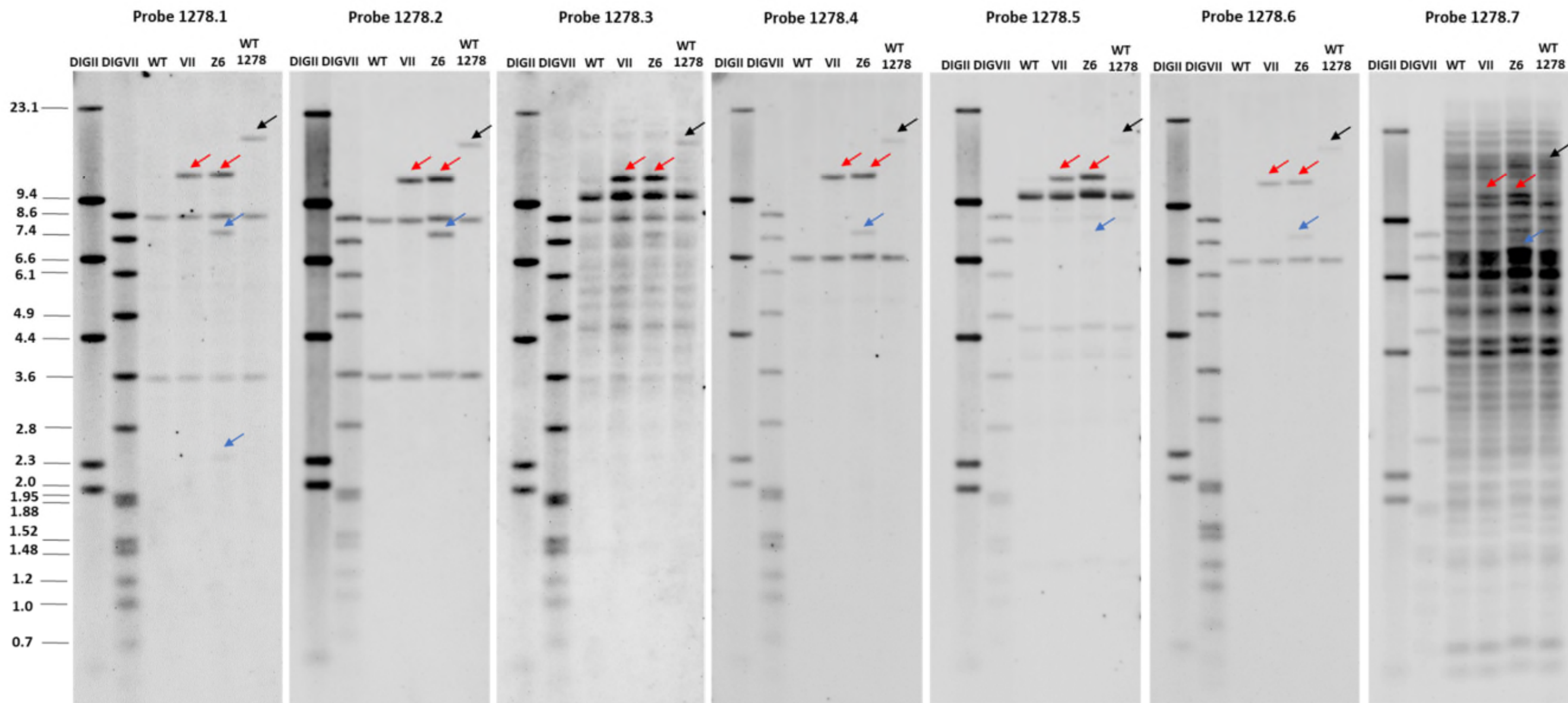


Figure 5. pSIM1278 and pSIM1678 Copy Number in Z6 Using pSIM1278 Probes

Southern blots of MfeI digested genomic DNA isolated from Snowden (WT), V11, Z6, and WT spiked with pSIM1278 plasmid DNA (WT p1278). Blots were hybridized with 7 unique pSIM1278 probes (1278.1–1278.7). In addition to endogenous bands common to all samples, three other types of bands were observed: bands unique to V11 and Z6 corresponding to the pSIM1278 insert (red arrow), bands unique to Z6 and corresponding to the pSIM1678 insert (blue arrow), and bands associated with spiking pSIM1278 plasmid DNA (**black** arrow) into the WT sample. The molecular weight markers, DIGII and DIGVII, were included in each gel and labeled in kilobases (kb) at the left of the first gel.

Internal bands were detected in Z6 and V11 samples indicating that the pSIM1278 T-DNA sequence between the EcoRV (2339) and ScaI (8233) cut sites was intact (Figure 6). Internal fragments with expected sizes resulted from the restriction digest with EcoRV, HindIII, EcoRI/ScaI, or PacI/XbaI (Figure 6). The 2.3 kb EcoRV, 4.2 kb HindIII and 3.8 kb EcoRI/ScaI fragments were detected by the AGP and GBS probes (Figure 7, Figure 8). A 5.3 kb PacI/XbaI fragment that hybridized to AGP and GBS probes, and a 1.3 kb PacI/XbaI fragment that hybridized to the GBS probe, were both detected (Figure 9, Figure 10). The 0.7 and 2.3 kb EcoRV fragments and a 4.2 kb HindIII fragment were observed when hybridized with the ASN probe (Figure 11). The 1.3 kb HindIII band and 3.8 kb and 0.8 kb EcoRI/ScaI bands were detected by the R1 probe (Figure 12). Both ASN and R1 probes are specific to pSIM1278 and did not hybridize to pSIM1678 sequences.

The analysis of junction fragments provided a means to confirm the presence of a single insert. Multiple inserts would result in an unexpected number of junction bands. The presence of MfeI sites in both left and right flanking regions made it possible to predict the sizes of the junction bands resulting from MfeI and XbaI digests (3.3 kb and 7.4 kb, respectively). Both fragments were detected in blots hybridized with the AGP probe (Figure 9). The 7.4 kb MfeI/XbaI junction band was also detected by the GBS probe (Figure 10). The junction bands provide support for the structure of the pSIM1278 insert as they overlap with internal fragments (Figure 6).

The flanking regions contain 1 kb of DNA that was confirmed by sequencing. However, the EcoRV, HindIII, EcoRI, and ScaI sites in the flanking regions were not identified, so the exact size of the junction bands resulting from restriction digestions using EcoRV, HindIII, EcoRI, and ScaI enzymes could not be predicted. The minimum sizes of the junction bands based upon known sequence and enzyme digestion sites were determined, as shown in Figure 6. In each case a corresponding band was identified in the appropriate Southern blot. The three left junction bands associated with EcoRV, HindIII and EcoRI/ScaI digests were identified exclusively by the AGP probe. The size of each band (5.3 kb EcoRV, 18 kb HindIII and 9 kb EcoRI/ScaI) was determined from observing how it ran on the gel, and consistent with expectations. Similarly, three fragments associated with the right junction (5.2 kb EcoRV, 8.6 kb HindIII, and 4.4 kb EcoRI/ScaI) were detected by the GBS and R1 probes (Figure 9, Figure 13) and all were consistent with their expected sizes (Figure 6). There were no unexpected bands that would suggest additional inserts of pSIM1278 T-DNA.

These data confirm that Z6 contains a single, nearly full-length T-DNA from pSIM1278.

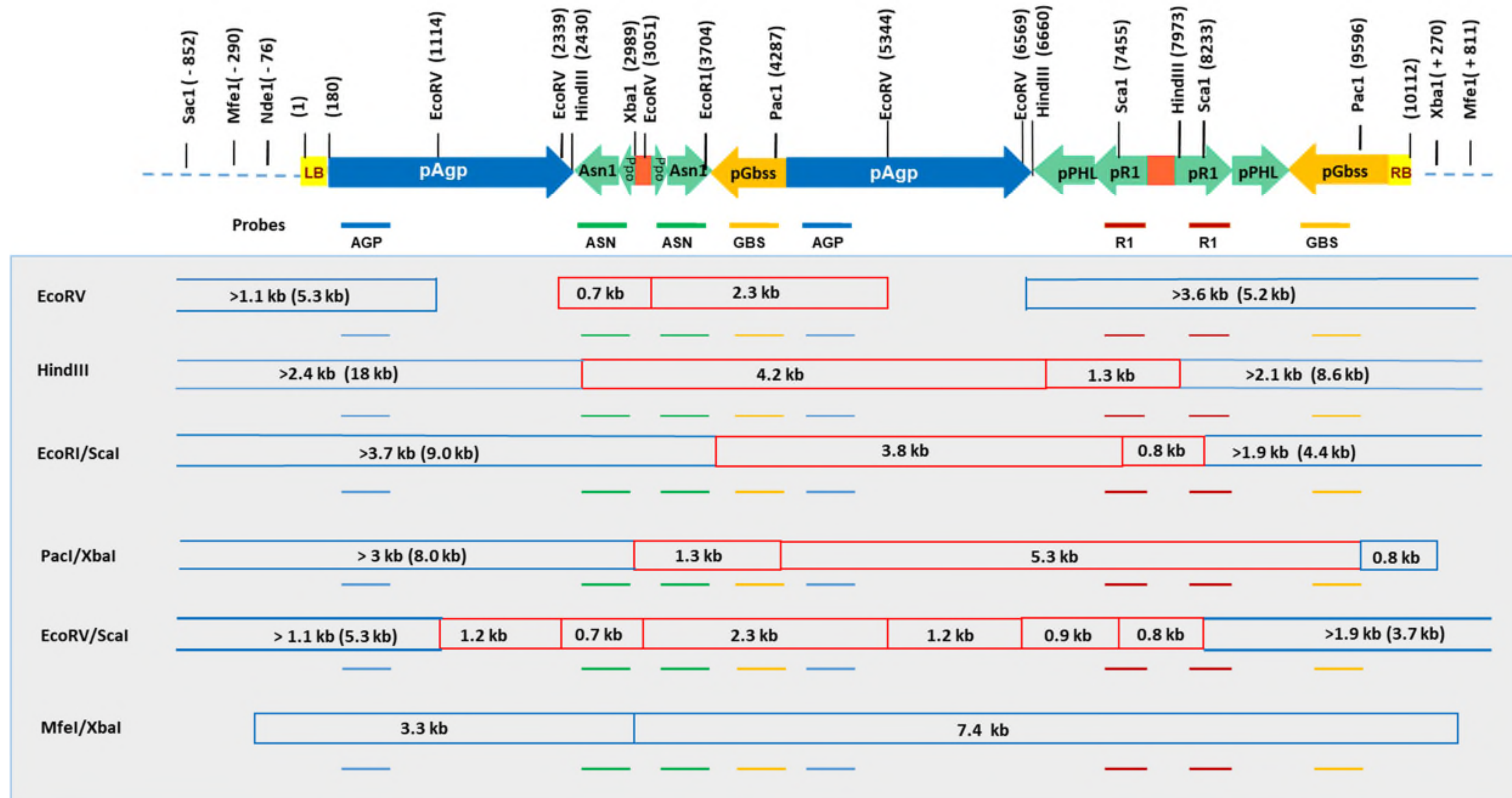
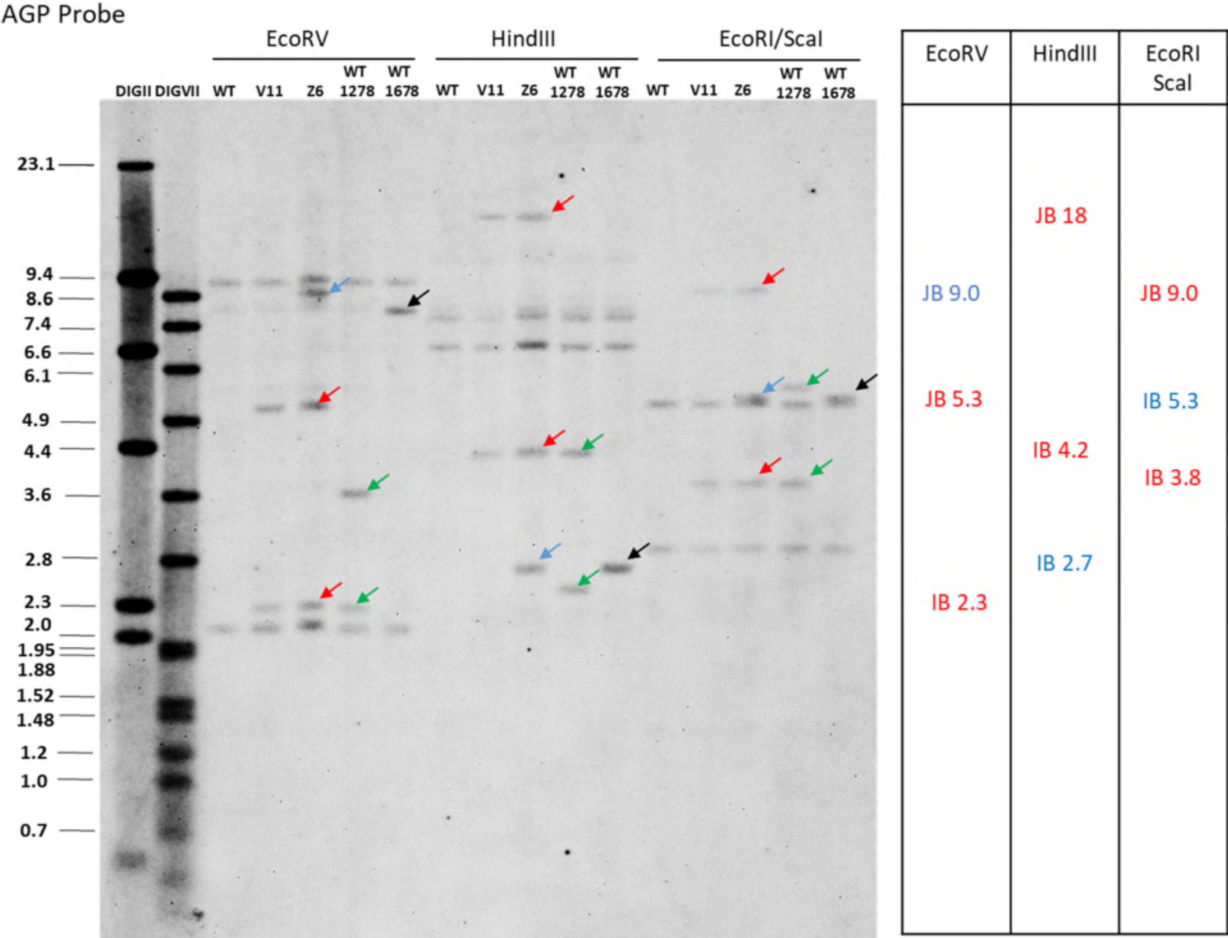


Figure 6. Structure of the pSIM1278 Insert in Z6 with Digestion Patterns and Probe Binding Sites

The figure represents the structure of the pSIM1278 insert in Z6, including restriction sites. The digestion pattern for selected enzymes is shown as colored boxes with the digest and fragment size indicated. The probes that hybridize to each digestion product are indicated below the fragment with colored lines. Red boxes denote internal bands and blue closed boxes indicate bands of known sizes due to identification of restriction sites within flanking DNA. Open-ended blue boxes indicate junction bands where the exact location of the second restriction site in the flanking region is unknown. The expected size for each these bands is indicated (i.e. distance to the end of the insert plus 1 kb to account for empirically-verified flanking region) along with the measured size based upon Southern blots (parentheses).



EcoRV	HindIII	EcoRI ScaI
	JB 18	
JB 9.0		JB 9.0
JB 5.3		IB 5.3
	IB 4.2	IB 3.8
	IB 2.7	
IB 2.3		

Figure 7. Southern Hybridization of Genomic DNA with AGP Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaII and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

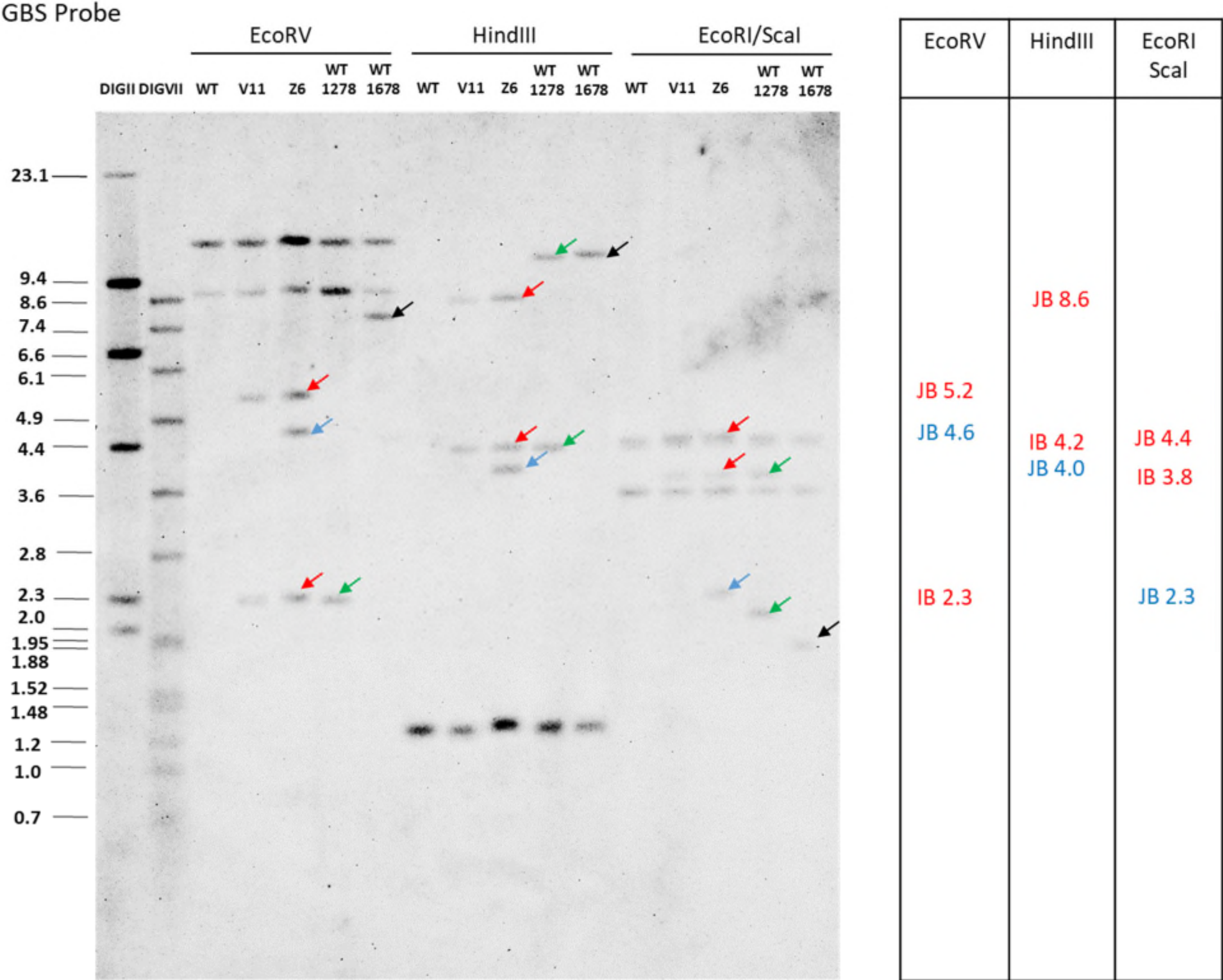


Figure 8. Southern Hybridization of Genomic DNA with GBS Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaII and hybridized with the GBS probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

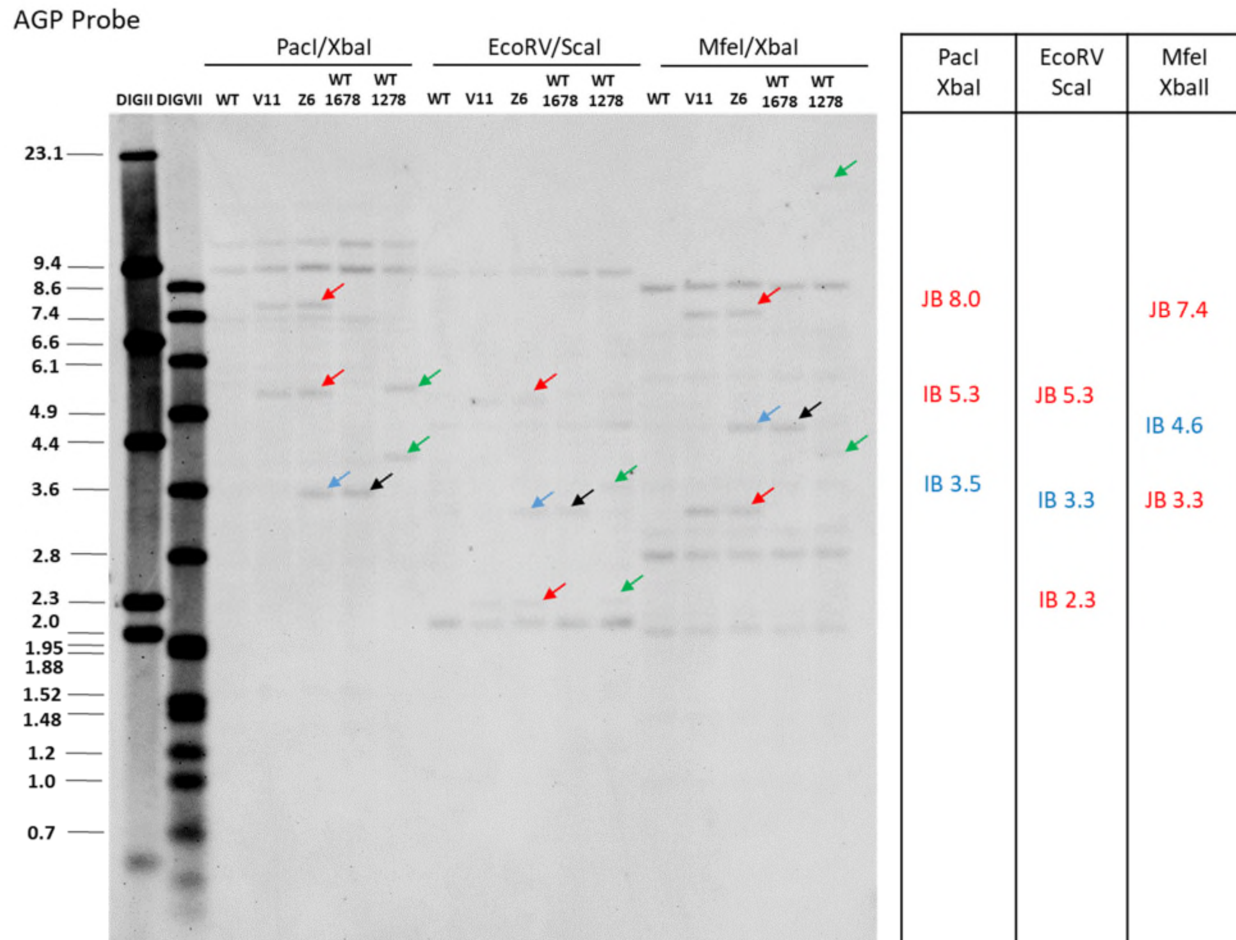


Figure 9. Southern Hybridization of Genomic DNA with AGP Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

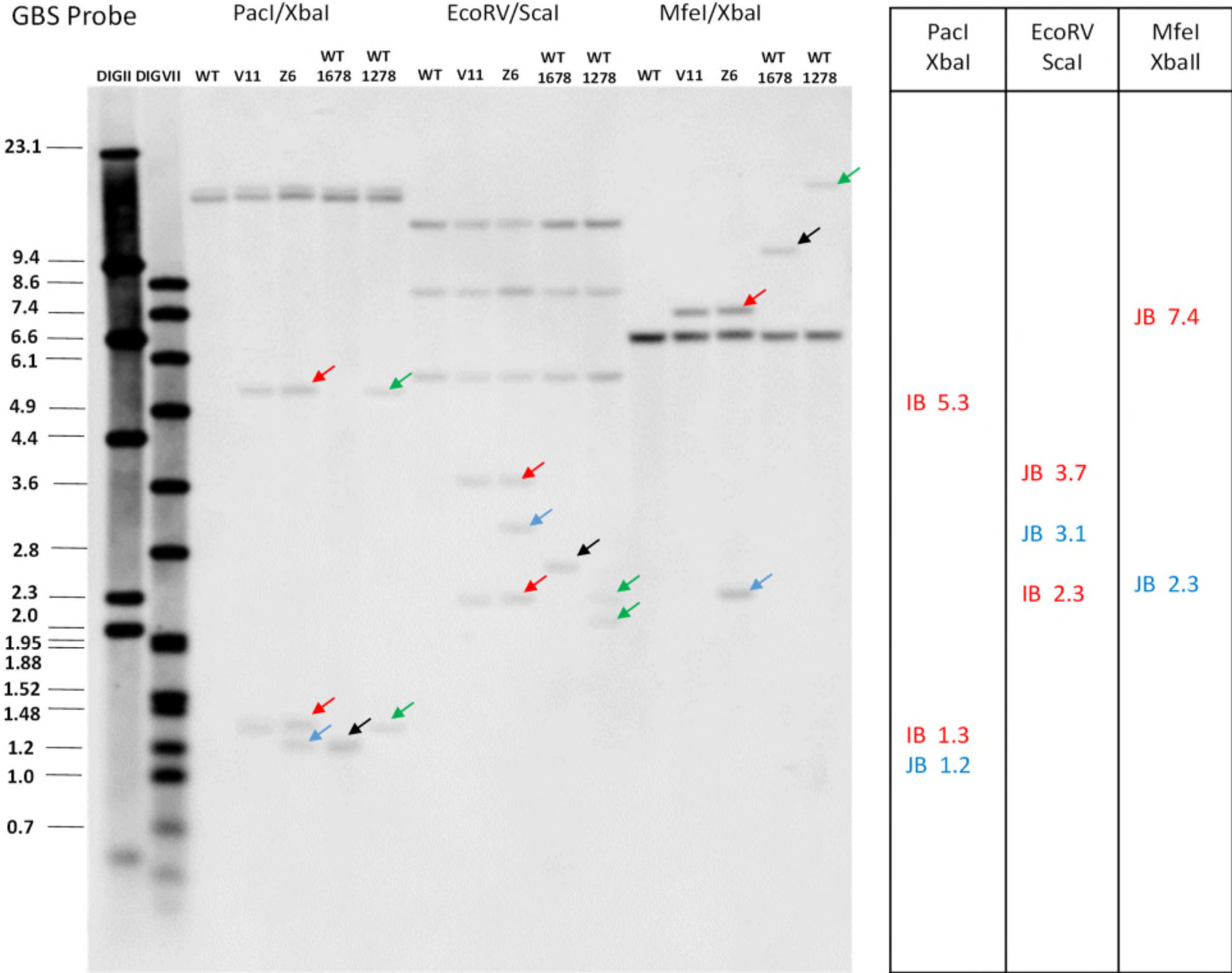


Figure 10. Southern Hybridization of Genomic DNA with GBS Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the GBS probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

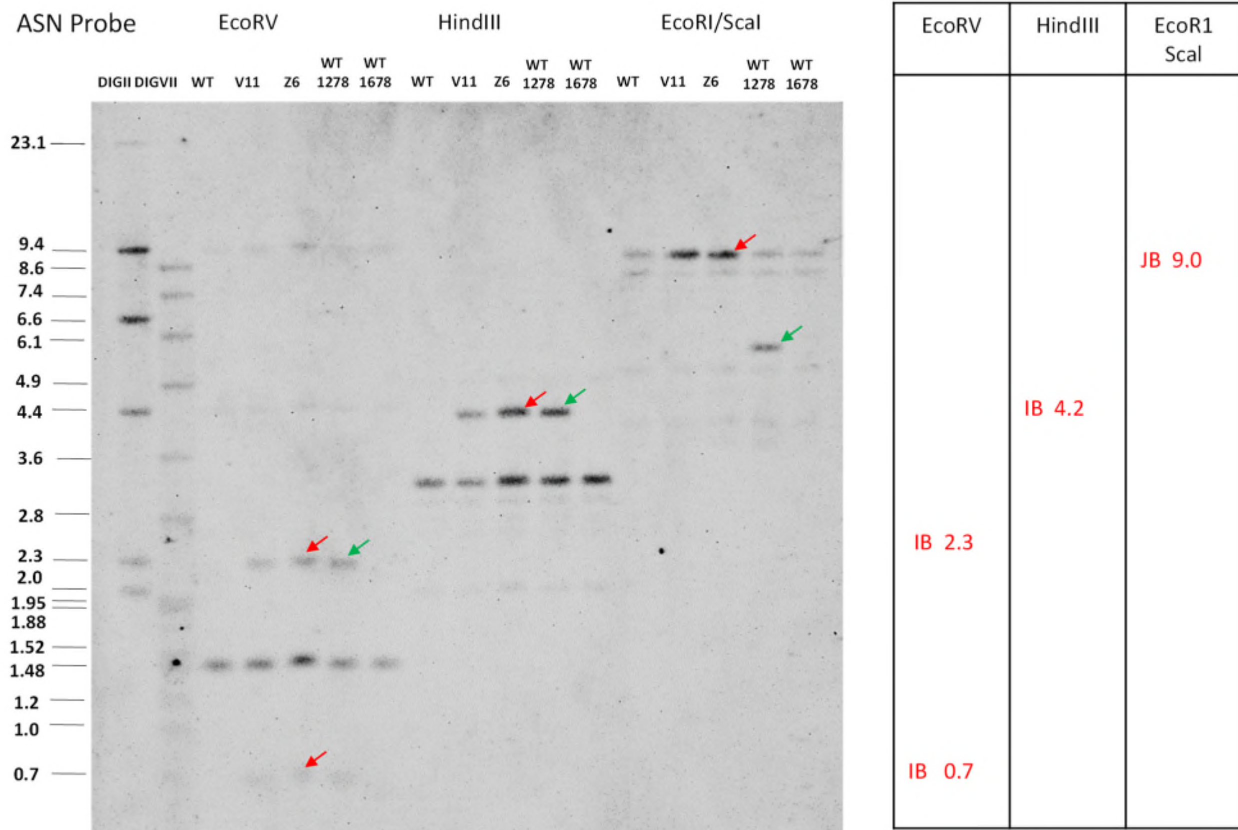


Figure 11. Southern Hybridization of Genomic DNA with ASN Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaII and hybridized with the ASN probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), and the spiked pSIM1278 plasmid (green). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

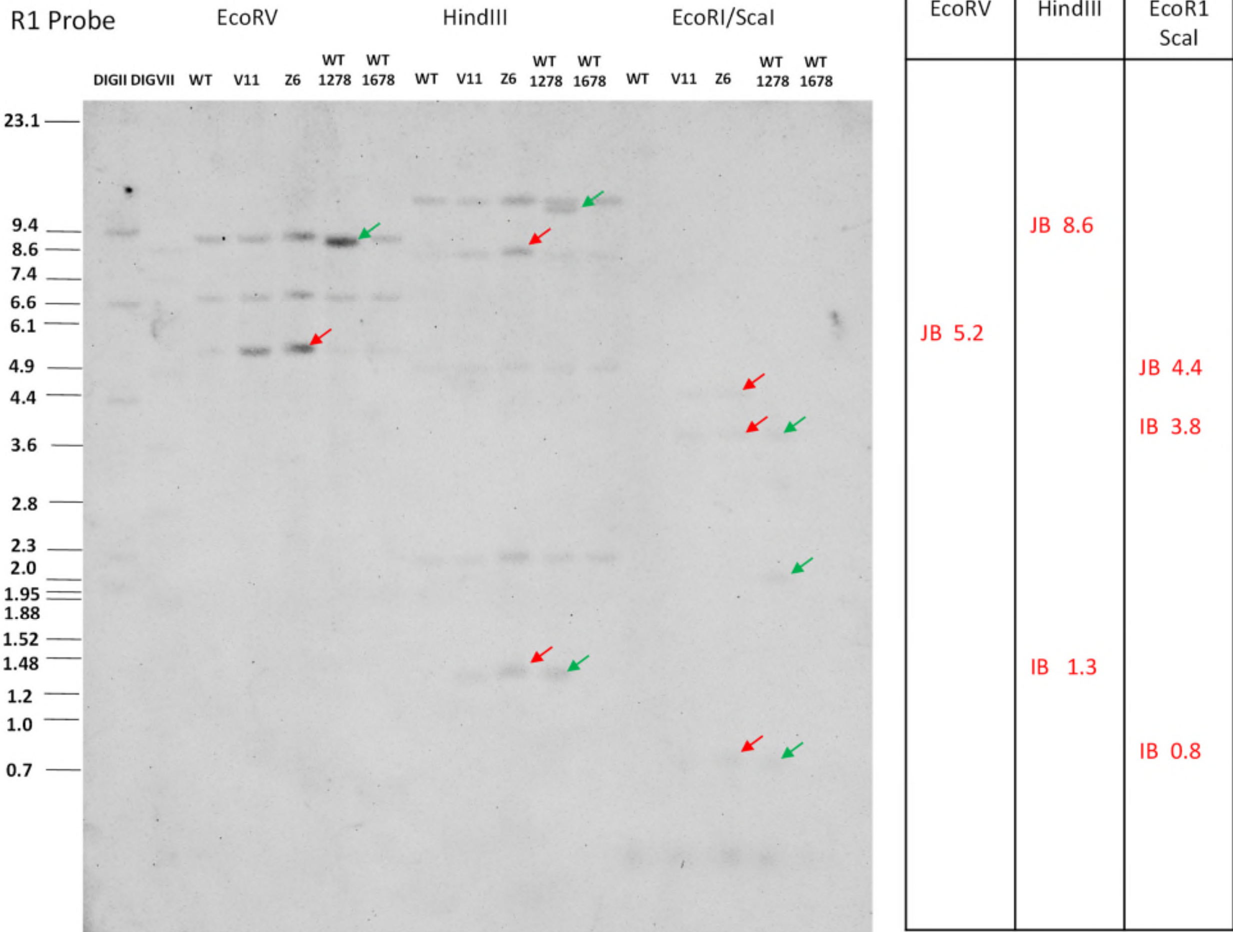


Figure 12. Southern Hybridization of Genomic DNA with R1 Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaII and hybridized with the R1 probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), and the spiked pSIM1278 plasmid (green). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

Z6 Insert from pSIM1678

The Southern analysis using MfeI digestion shown above determined the pSIM1678 insert in Z6 consists of a single, full-length copy (Figure 5). The junctions between the pSIM1678 insert and the plant genome were determined using DNA sequencing. Additional Southern blot analysis was conducted to confirm the pSIM1678 insert structure. The pSIM1678 insert structure was characterized like the pSIM1278 insert with additional probes (VNT and INV). The structure of the insert, restriction sites and digestion products, and probe binding sites are summarized in Figure 13. Like the pSIM1278 characterization, the internal bands (IB) are shown in red and junction bands (JB) in blue with open and closed boxes. The expected size of each band was provided for cross-reference with the Southern blot analysis. All the internal bands and junction bands detected in Z6, but not in WT or V11, are associated with the pSIM1678 insert.

To determine pSIM1678 insert structure, internal bands were analyzed first because their size and copy number are predictable for a simple insert (Figure 13). The Southern blots probed with AGP and GBS (Figure 7-10) were evaluated as the pSIM1278 and pSIM1678 T-DNA both contain *Agp* and *Gbss* promoters. Three internal pSIM1678 T-DNA fragments (3.5 kb PacI/XbaI, 4.6 kb XbaI, and 5.3 kb EcoRI/ScaI) were detected by both AGP and INV probes (Figure 7, 9 and 14). The 2.7 kb HindIII and 3.3 kb EcoRV/ScaI fragments were detected by the AGP probe (Figure 7, Figure 9). The 1.2 kb PacI/XbaI fragment were also detected using the INV probe (Figure 14), which hybridizes to pSIM1678, but not pSIM1278.

Similar to the pSIM1278 insert, the junction regions for the pSIM1678 insert adjoin the plant genome to the left and right border regions (LB and RB) of the insert. Restriction sites were identified on each flanking region (Figure 13).

The VNT probe was designed to hybridize to the promoter region of *Rpi-vnt1* and binds near the left border of pSIM1678 T-DNA, which makes it ideal for detecting the left junction fragments. Three restriction sites (XbaI, ScaI, and MfeI) were identified on the flanking region near left border of pSIM1678 T-DNA. The 3.9 kb XbaI, 1.1 kb ScaI and 2.4 MfeI junction fragments detected by the VNT probe (Figure 15) connect the left side of the pSIM1678 insert with the flanking region as depicted in Figure 13.

The right junction DNA sequencing revealed restriction sites for MfeI, ScaI, and PacI, which can be used to characterize the right side of the insert using the INV and GBS probes. Unlike the GBS probe, the INV probe is specific to the pSIM1678 insert. The 3.1 kb EcoRV/ScaI, 2.3 kb XbaI/MfeI and 2.3 kb EcoRI/ScaI junction bands were detected in Z6, not WT and V11 samples, by INV probe as expected (Figure 14, Figure 16). Southern with XbaI/PacI digested DNA of Z6 resulted in two internal fragments (3.5 and 1.2 kb) and both were observed using the INV probe (Figure 14). The XbaI/MfeI and EcoRI/ScaI digests bisect the VInv cassette resulting in an internal band and a junction band for each digest. The XbaI/MfeI (4.6 kb IB and 2.3 kb JB) and EcoRI/ScaI (5.3 kb IB and 2.3 kb JB) bands were detected by the INV probe (Figure 14, Figure 16) confirming the presence of partial Vnt1 cassette and a complete copy of the VInv inverted repeat cassette on the right side.

These data indicate that Z6 consists of a single, nearly full-length T-DNA from pSIM1678.

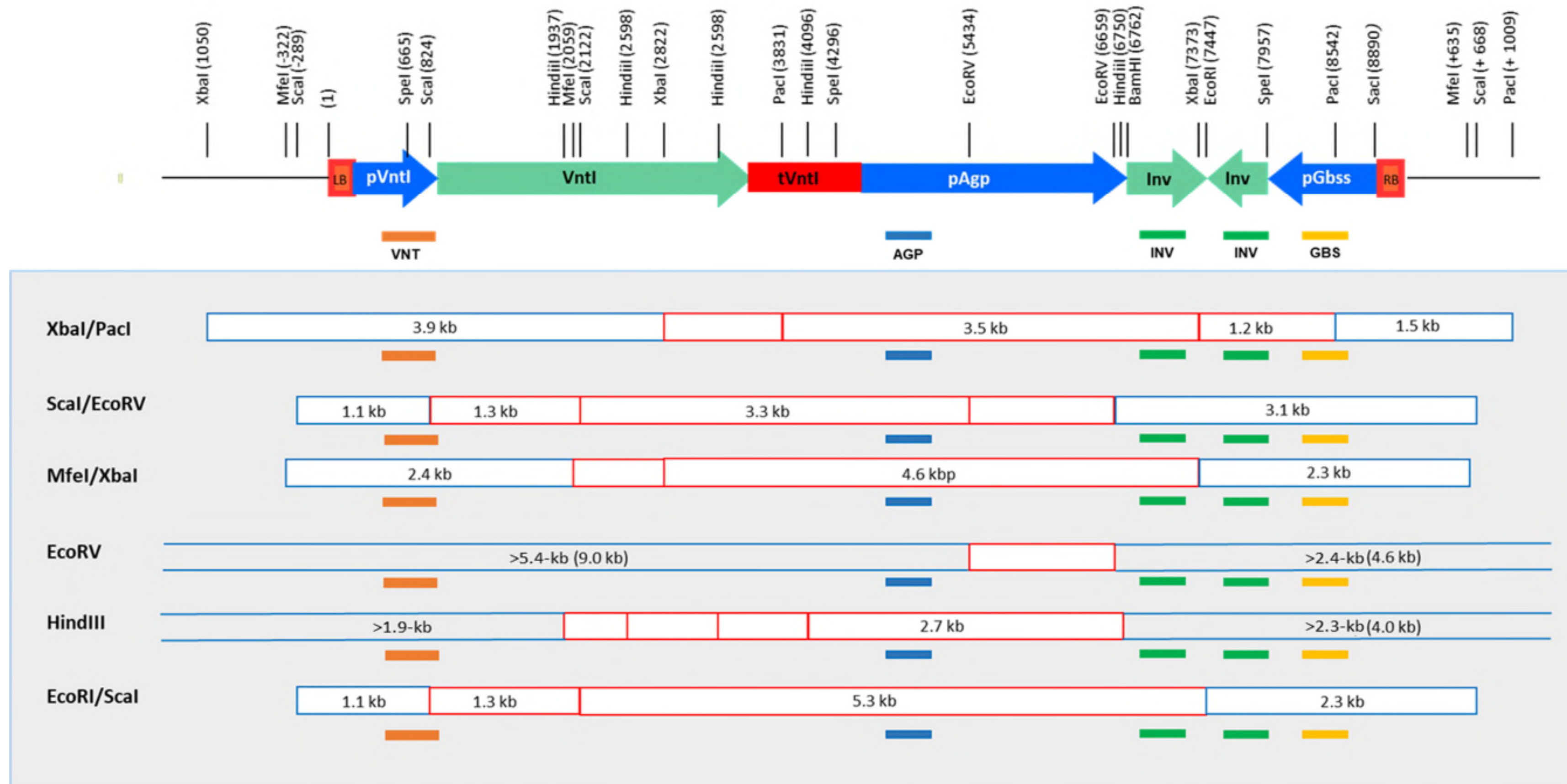


Figure 13. Structure of the pSIM1678 Insert in Z6 with Digestion Patterns and Probe Binding Sites

The figure represents the structure of the insert associated with the pSIM1678 construct, including designated restriction sites. The digestion pattern for selected enzymes is shown as colored boxes with the digest and fragment size indicated. The probes that are expected to detect each digestion product are indicated below the fragment with colored lines. All expected probe binding sites are indicated, but only the digest/probe combinations necessary to support the model are provided. Red boxes denote internal bands (IB) associated with the pSIM1678 DNA construct. Blue closed boxes indicate bands of known sizes due to identification of restriction sites within flanking DNA. Open-ended blue boxes indicate junction bands where the exact location of the second restriction site on flanking region is unknown. The expected size for each these bands is indicated (i.e. distance to the end of the insert plus 1 kb to account for empirically-verified flanking region) along with the measured size based upon Southern blots (parentheses).

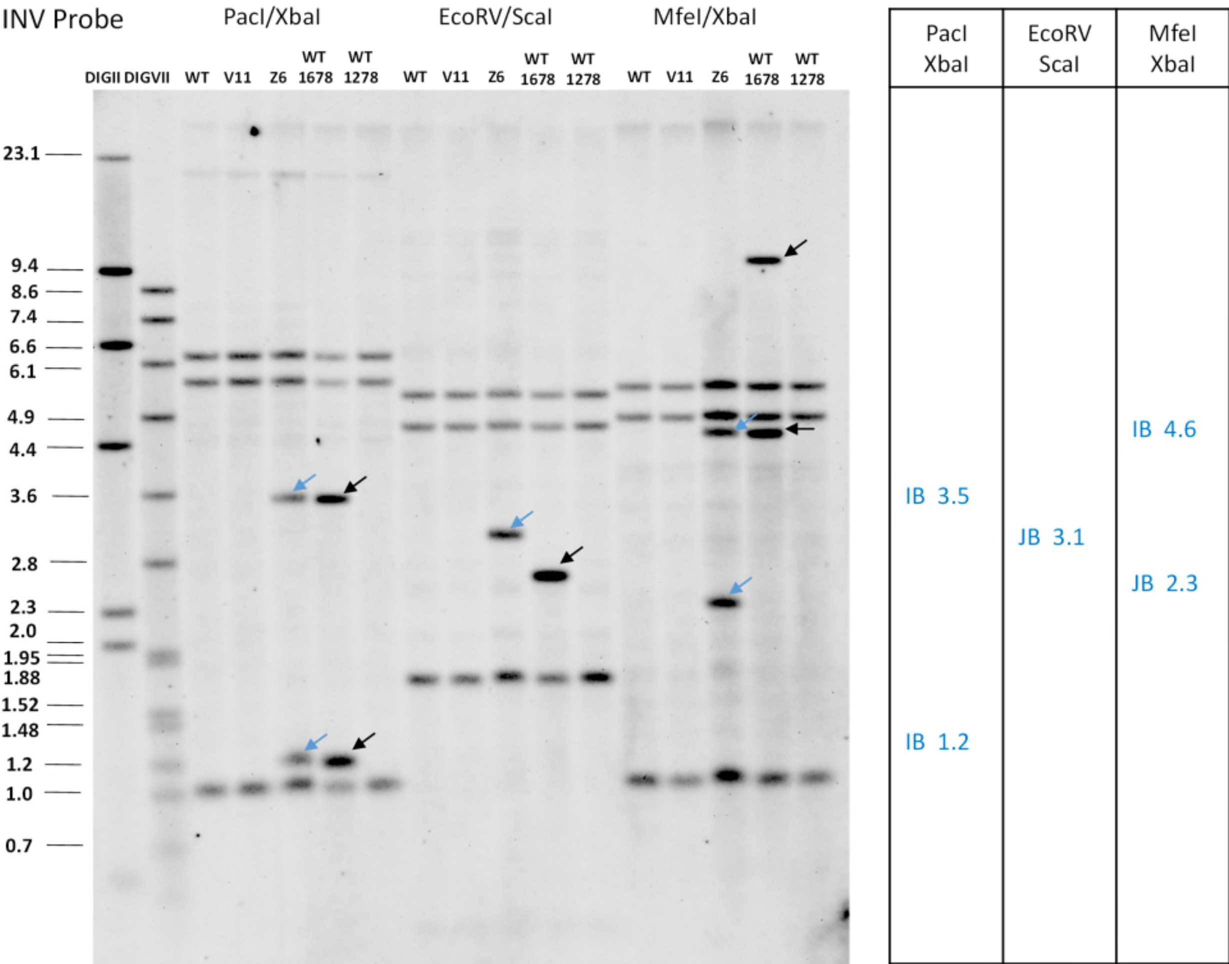
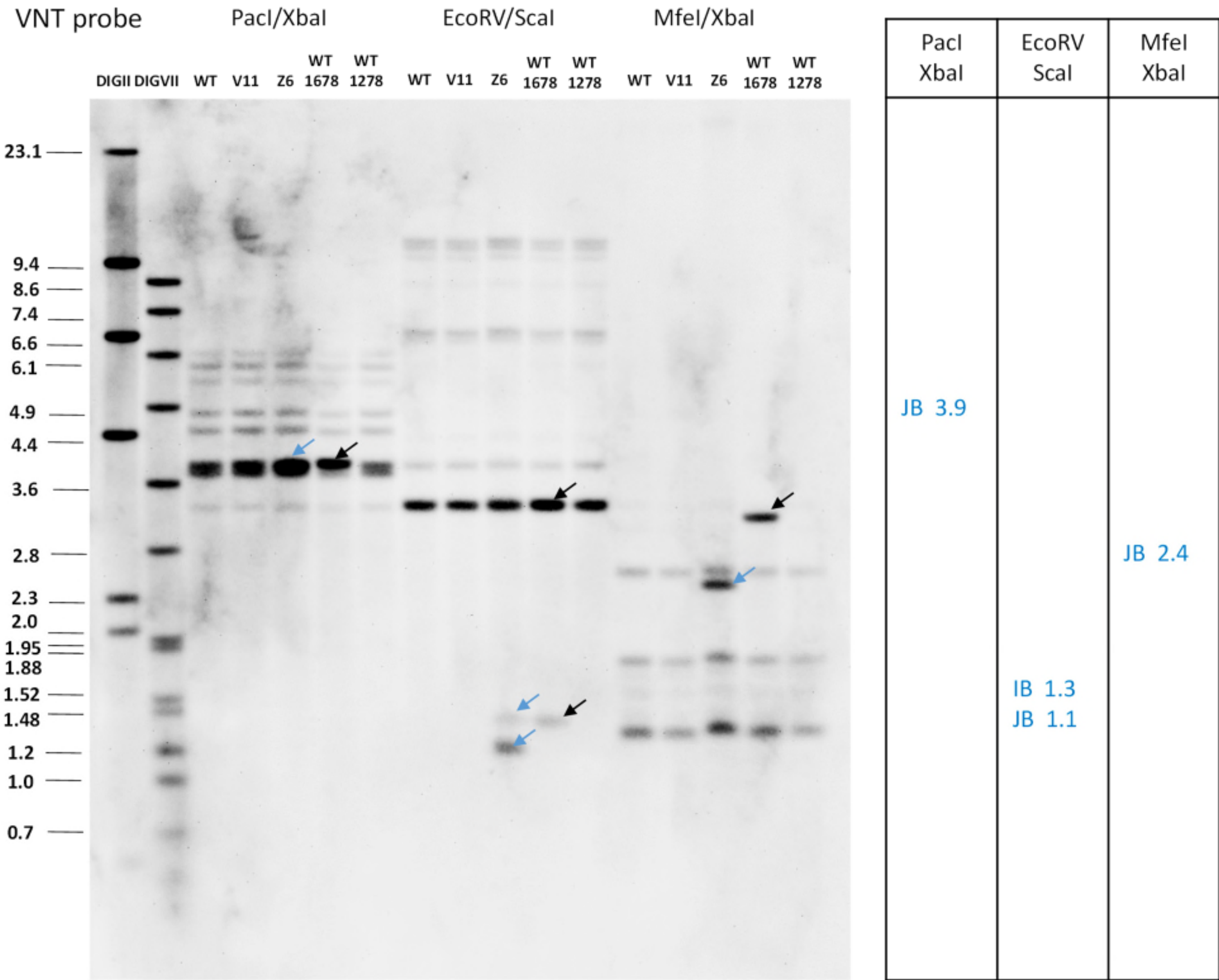


Figure 14. Southern Hybridization of Genomic DNA with INV Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with PacI/XbaI, ScaI/EcoRV, and XbaI/MfeI and hybridized with the INV probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1678 insert (blue) and the spiked pSIM1678 plasmid (black). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).



PacI XbaI	EcoRV ScaI	MfeI XbaI
JB 3.9	IB 1.3 JB 1.1	JB 2.4

Figure 15. Southern Hybridization of Genomic DNA with VNT Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with PacI/XbaI, ScaI/EcoRV, and XbaI/MfeI and hybridized with the VNT probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1678 insert (blue) and the spiked pSIM1678 plasmid (black). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

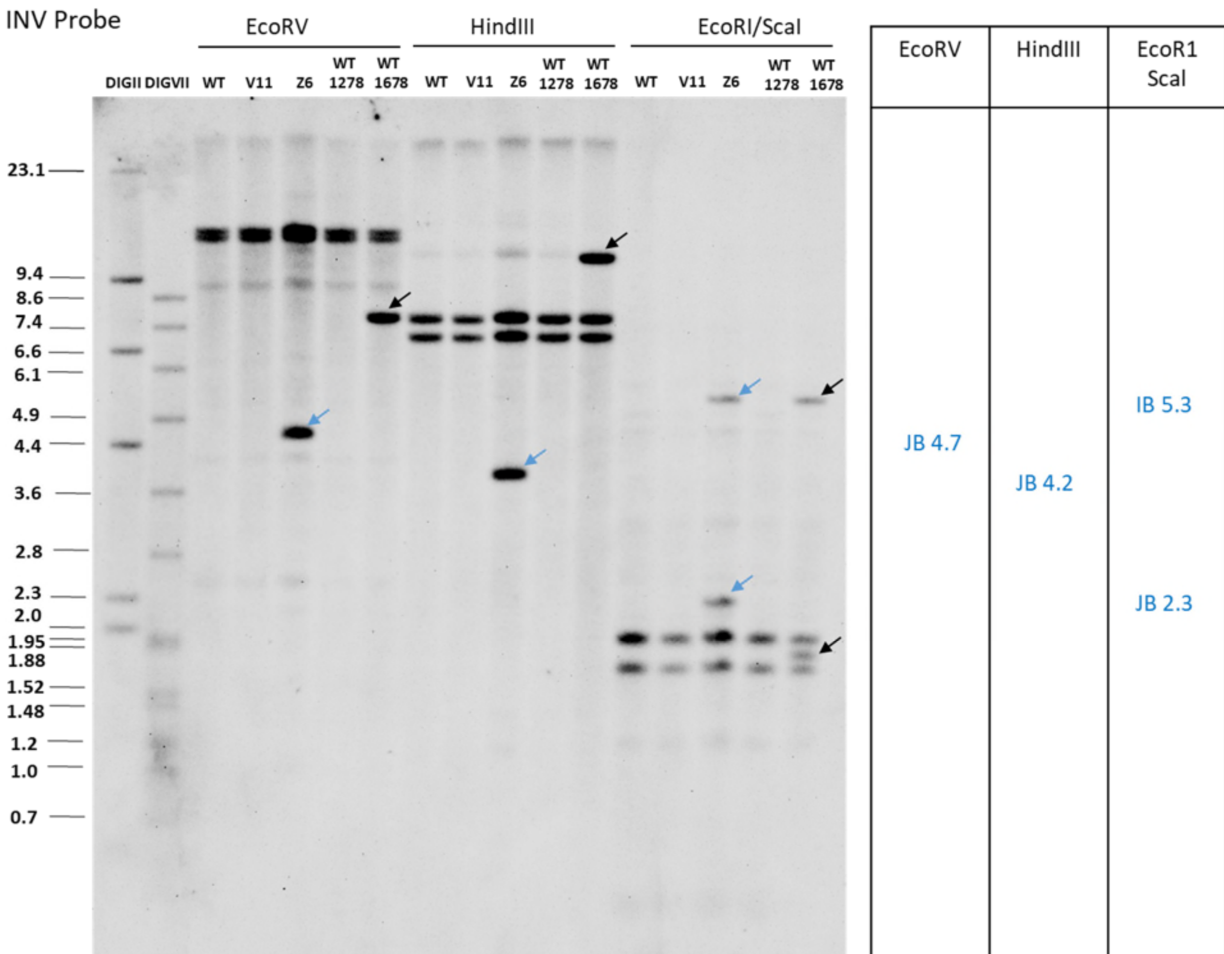


Figure 16. Southern Hybridization of Genomic DNA with INV Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the INV probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1678 insert (blue) and the spiked pSIM1678 plasmid (black). The estimated sizes of insert-specific bands are summarized in the associated table using the same coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

Conclusion

A combination of Southern blot and DNA sequencing analysis (data below) was used to show that Z6 contains single, nearly full-length inserts from both pSIM1278 and pSIM1678. The Southern blot data generated for the pSIM1278 insert in Z6 matched the previous Southern blot data generated for its V11 parent. The transformation of V11 with pSIM1678 resulted in a single T-DNA integration in Z6, which consists of a single Rpi-vnt1 expression cassette and a single vacuolar invertase down-regulation cassette.

Characterisation of the pSIM1278 and pSIM1678 Inserts in Z6

Molecular characterization of the pSIM1278 and pSIM1678 inserts in Z6 were conducted with a combination of methods including droplet digital PCR [unpublished report], captured mate-pair Illumina sequencing, Southern blotting (see above and [unpublished report]), PCR, and Sanger sequencing. Further details are provided in [unpublished report].

Number of Insert Validation

Research using Illumina Next Generation Sequencing (NGS) data to characterize transgene inserts suggests that this method can be used to replace Southern blots (Zastrow-Hayes et al., 2015). Here, a similar approach was used in conjunction with Southern blot analysis. Mate pair libraries and multiplexed targeted sequencing were used to adapt to the challenges of identifying inserts containing native, potato sequences and inverted repeats. Insertion locus identification with bioinformatic scripts indicated one insert each from pSIM1278 and pSIM1678 in the Z6 genome.

Insert Flanking Sequences

The pSIM1278 T-DNA insert and the pSIM1678 T-DNA insert in Z6 each consists of a single locus as indicated by ddPCR, next generation sequencing analysis, and Southern blotting (see above and [unpublished report]). PCR generated products for Sanger sequencing across the junctions near the left border (LB) and right border (RB) of each insert including at least 1 kb of flanking DNA (Table 1 in [unpublished report]). After Sanger sequencing of the left and right junction regions, a reference sequence for the pSIM1278 insert and flanking sequence (depicted in Figure 17A) and a reference sequence for the pSIM1678 insert and flanking sequence (depicted in Figure 17B) were assembled for validation by Illumina data.

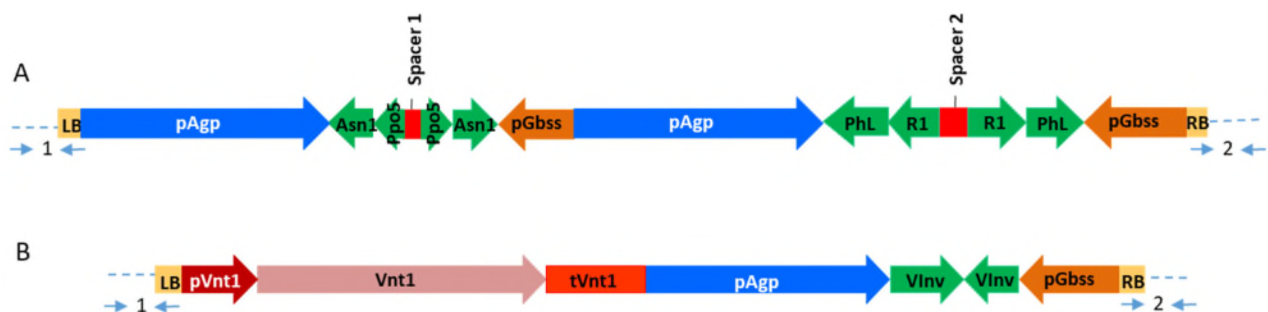


Figure 17. Insert Structures in Z6 with Junctions Labeled

The Z6 event contains a single, nearly full-length T-DNA from (A) pSIM1278 insert and (B) pSIM1678 insert. The plant genomic flanking sequence is indicated with a dashed line. Arrows denote primer pairs (Table 1 in [unpublished report]) used to amplify the junction near the left border (LB, 1) and the right border (RB, 2) for each insert.

Insert Sequence Validation

Illumina data were used to validate the insert structure that was determined by Southern blotting (see above and [unpublished report]). The Z6 Illumina library was generated by sequencing mate pair DNA fragments sharing sequence identity to either pSIM1278 or pSIM1678 after targeted capture. As described above, the reference pSIM1278 insert (event V11) and the reference pSIM1678 insert were assembled from Sanger sequenced flanking genomic junctions and the T-DNA sequence. Illumina mate pair sequencing reads were aligned to both references as well as the genome. Targeted capture and resulting enrichment yielded thorough depth of coverage of read pairs across the entire pSIM1278 insert, including the junctions and adjacent flanking genomic DNA (Figure 18A) and the entire pSIM1678 insert, including the junctions and adjacent flanking genomic DNA (Figure 18B). The alignments were inspected to confirm accuracy by depth of coverage and sequence quality scores across the entire pSIM1278 insert and pSIM1678 insert.

Sequence data (Appendix A in [unpublished report]) showed that the pSIM1278 insert contains a nearly full-length T-DNA with a 14 bp deletion from the left border annotation and a 23 bp deletion from the right border annotation. Sequence data (Appendix B in [unpublished report]) showed that the pSIM1678 insert contains a nearly full-length T-DNA with a 9 bp deletion from the left border annotation and a 36 bp deletion from the right border annotation.

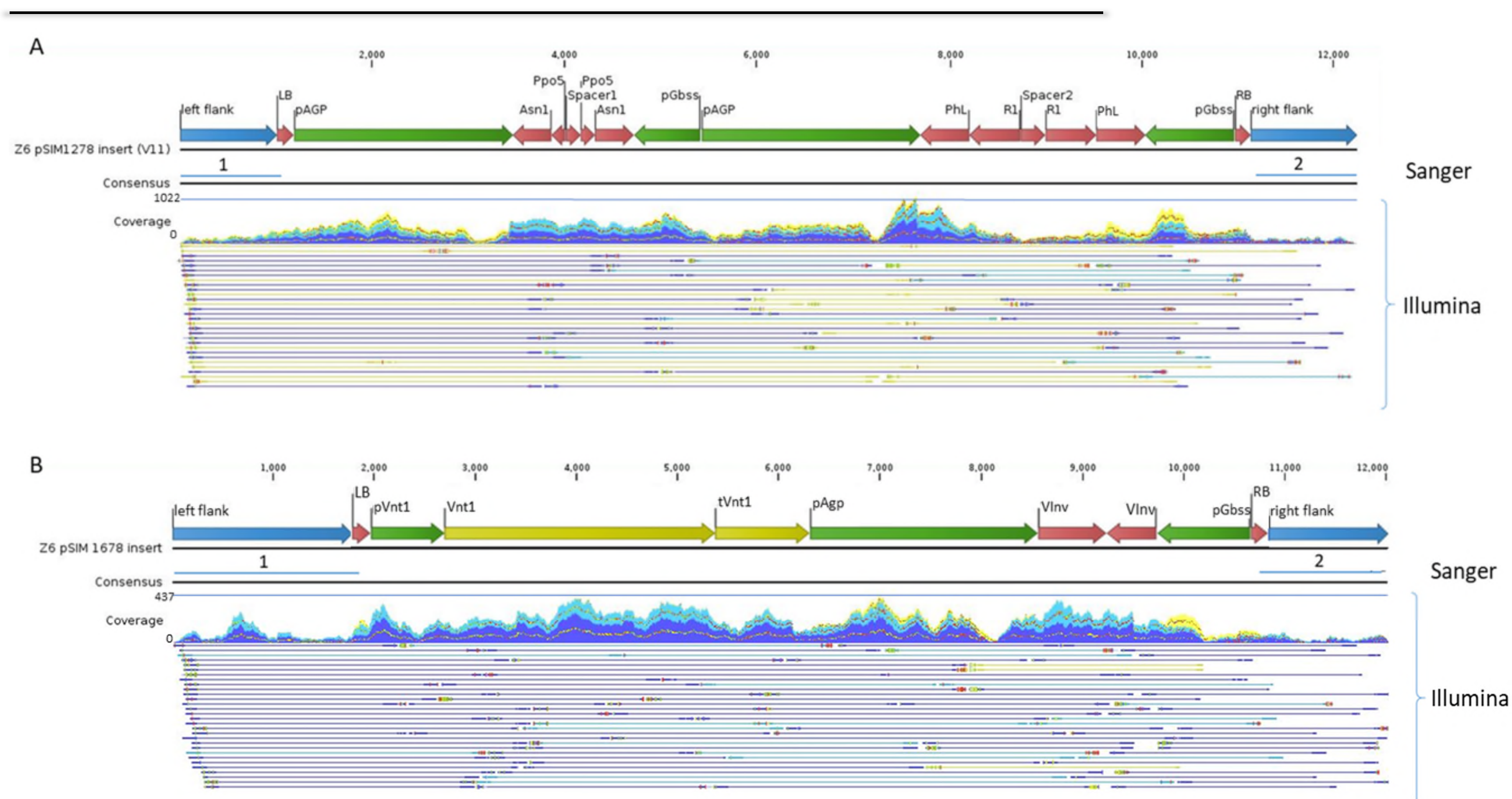


Figure 18. Summary of the Sequence Characterization of the pSIM1278 and pSIM1678 Inserts

Maps of the (A) pSIM1278 insert (V11) and (B) pSIM1678 insert are shown with blue lines used to represent regions Sanger sequenced, where 1 is the left junction and 2 is the right junction. Illumina sequence coverage depth of paired reads is displayed, indicating that there are more reads than shown. Yellow corresponds to reads that align to multiple places on the inserts (i.e. inverted repeats or duplicated promoters).

Insertion Site Characterisation

Agrobacterium-mediated transformation results in T-DNA being introduced at one or more random locations in the genome. Snowden potatoes are tetraploid, containing four homologous sets of chromosomes, and each insert is expected to occur at a unique locus on one of the four homologues. Therefore, Z6 retains three chromosomes with a native locus homologous to the insertion site following transformation.

The sequence of the genomic integration site for the insert from pSIM1278 was determined using primers that hybridize to the flanking genomic regions of the insert (Figure 19). PCR with these primers amplified the homologous, native loci in untransformed Snowden, resulting in an amplicon of approximately 1.4 kb (Table 1 in [unpublished report]). Comparison of the Sanger sequenced insertion site (Appendix C in [unpublished report]) and the insert described above (Appendix A in [unpublished report]) indicated that 72 bp of genomic DNA was deleted as a result of the T-DNA insertion from pSIM1278. BLAST of the 1.4 kb sequence aligns to a region of the potato reference genome chromosome 4 (region 63937636-63939054) with 97% ID. The insertion site (72 bp deleted sequence highlighted purple, Figure 19B) does not indicate interruption of known genes.

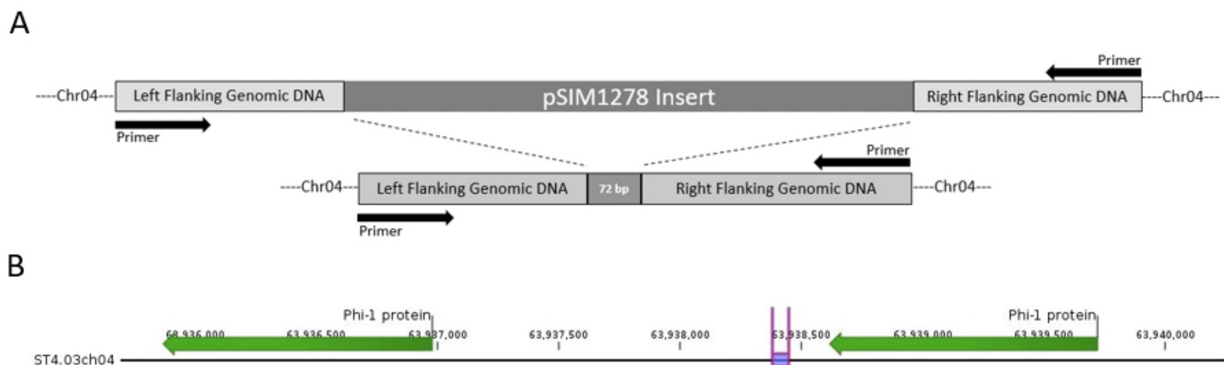


Figure 19. Characterization of the pSIM1278 Insertion Site on Chromosome 4

(A) Primers were designed using the left and right flanking regions of the insert (top) to amplify the native locus (bottom). Sanger sequence of the amplicon showed that 72 bp of genomic DNA was deleted upon insertion of the T-DNA. (B) The region deleted by the T-DNA insertion (purple highlight) occurs between two gene annotations in the reference genome.

The sequence of the genomic integration site for the insert from pSIM1678 was determined using primers that hybridize to the flanking genomic regions of the insert (Figure 20). PCR with these primers amplified the homologous, native loci in Snowden. Due to a duplication of 957 bp at the left and right flanking sequence, primers were designed outside using sequence unique to the left and right flank. After Sanger sequencing, identity of the insertion site (Appendix D in [unpublished report]) was annotated relative to the insert described above (Appendix B in [unpublished report]). A BLAST search of the 957 nt duplicated sequence at each flank aligns to a region of the potato reference genome chromosome 9 with 97% ID (region 3678695-3677323), which does not indicate interruption of known genes (Figure 20B).

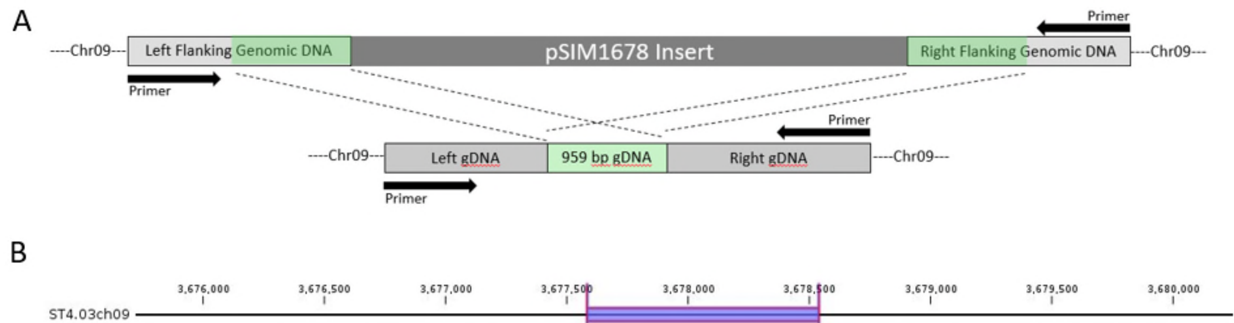


Figure 20. Characterisation of the pSIM1678 Insertion Site on Chromosome 9

(A) Primers were designed using the left and right flanking regions of the insert (top) to amplify the native locus (bottom). Sanger sequence of the amplicon showed that 957 bp of genomic DNA (light green) was duplicated on insertion of the T-DNA. (B) The region duplicated at both flanks is highlighted (purple) on the annotated reference genome in a region without annotated genes.

Absence of Vector Backbone DNA

Illumina mate pair sequencing libraries were captured to enrich for high coverage of sequences derived from pSIM1278 or pSIM1678, including the backbone (which is shared between the two vectors). The backbone contains two elements comprised of potato DNA, the *Ubi7* promoter and the *Ubi3* terminator, which were the only regions of backbone that contained mapped reads (Figure 21). The mate pairs of these reads were inspected and do not connect to either of the pSIM1278 or pSIM1678 insertion site loci. The two reads that map to the backbone are most likely derived from common laboratory high copy plasmid DNA. The results are comparable to the Snowden conventional control. These results indicate that no backbone is inserted in Z6, corroborating Southern blot data (see [unpublished report]).

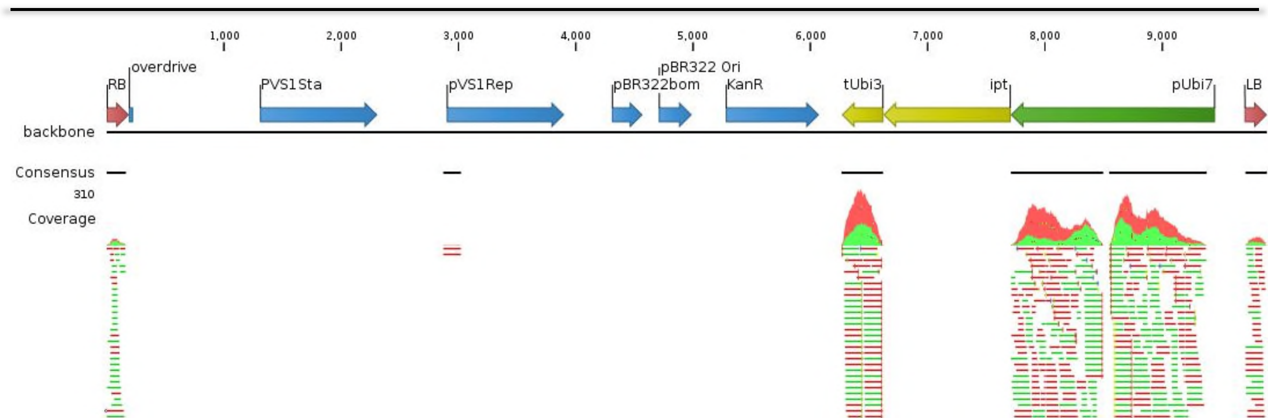


Figure 21. Illumina sequencing reads aligned to the binary vector backbone

Potato DNA derived elements Ubi7pro, Ubi3T, and the T-DNA borders show significant representation in the Z6 enriched sequencing library, as well as in the untransformed Snowden control (not shown). Red and green lines are forward and reverse reads, respectively.

Conclusion

A combination of Sanger and Illumina NGS sequencing corroborated studies using ddPCR and Southern blots and showed the presence of single inserts associated with transformation of Z6 using pSIM1278 and pSIM1678. The structure and sequences of the two inserts in Z6 are provided, with flanking DNA sequence. No backbone DNA was integrated into the Snowden genome. No annotated genes were disrupted by the insertion of these T-DNAs.

A.3(c)(iv) A map depicting the organisation of the inserted genetic material at each insertion site

Details of the organisation of the inserted genetic material at each integration site are described above. Specifically:

1. **Event Z6**–Detailed organisation of the genetic elements in each insert (Figure 18), integration of pSIM1278 (Figure 19) and pSIM1678 (Figure 20).

A.3(c)(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs)

The sequences of the ORFs covering the junctions between the inserts and the potato flanking regions were evaluated as part of the ORF analysis (see Section B1(d) and [unpublished report]). None of the junction ORFs were identified as homologs of known toxins or allergens (see Section B1(d)).

A.3(d) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process) including which generations have been used.

Potato plants are cultivated by vegetative propagation and commonly maintained as disease-free plantlets in tissue culture. Shoots from the plantlets are cut and transferred to fresh medium periodically to maintain healthy stocks (Figure 22). When many plants are needed, for example for seed production, multiple shoots are cut and grown in tissue culture medium. Plantlets with roots are transferred to greenhouses to produce tubers for seed. Greenhouse tubers are planted in fields to multiply potato seed for large-scale potato production.

Potato seed is a tuber that contains buds, called "eyes", which sprout and grow into mature potato plants. The seed tuber is planted whole or as a cut piece with eyes (Figure 22).

In vegetative (asexual) propagation, progeny arise from a single parent plant, and each progeny cell receives the same genetic material. As the parent cell divides in two, the resulting progeny cells are copies of one another and the parent plant. Vegetative propagation produces a genetically uniform crop because no new genetic material is introduced through sexual reproduction.

During seed production growers eliminate plants in the field that are "off-types" in order to maintain the desired characteristics of the variety. In addition, commercial seed is reinitiated from the disease-free, tissue culture source material on a continuous basis.

As a result of vegetative propagation, each potato is a genetic clone of its parent plant since the tuber (and not the true seed) is used to generate the next plants (Figure 23). An example of potato vegetative propagation is shown in (Figure 23). Tuber production begins with parent plants containing the desired traits of interest. Cuttings from the parent plants are propagated in tissue culture (plantlets). These are transferred to greenhouses to grow mini-tubers for distribution to seed farmers. Mini-tubers are planted in fields to produce tuber seed. After multiple seasons of re-planting tubers (generally 3–5 seasons), the tubers will be sold for commercial potato production and seed production reverts to tissue culture parent plants that have been tested to confirm the presence of the desired traits and disease-free status.

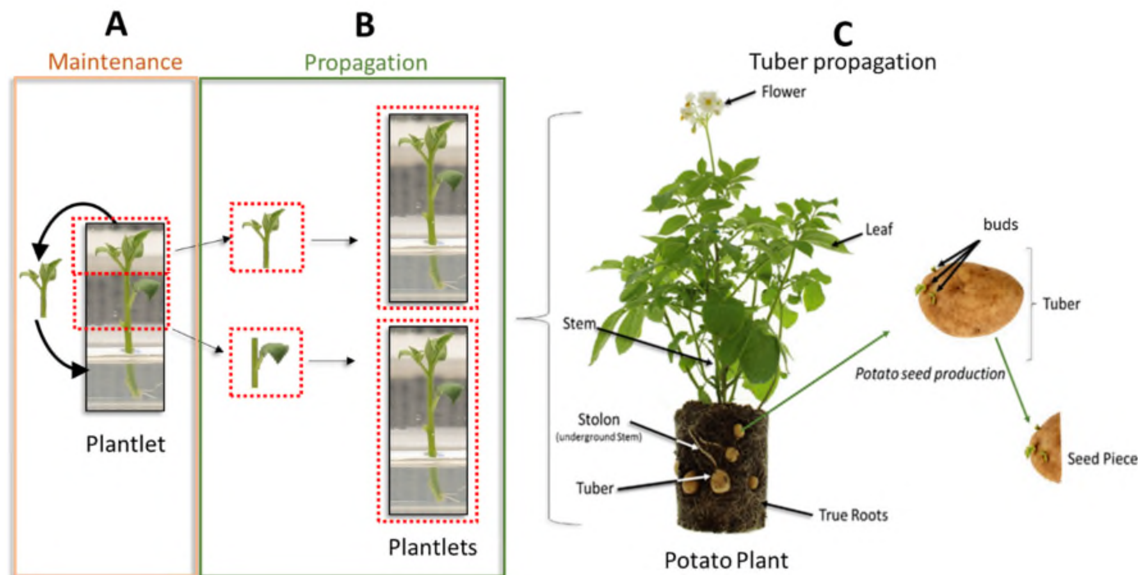


Figure 22. Potato Plant and Tuber Propagation

Potato variety stocks are maintained in tissue culture (A) and multiplied by vegetative propagation (B) to produce mature potato plants and potato seed (C).

Figure 23. Commercial Production of Potatoes

Plantlets are propagated from cuttings of the stock tissue culture plantlet. Plantlets are transferred to soil or grown using Nutrient Film Technique (NFT) or hydroponics in greenhouses. Tubers from these Greenhouse-0 plants are referred to as mini-tubers. An entire mini-tuber is planted in either the greenhouse or field to produce a new potato plant. Tubers from Greenhouse-1/Field Grown-1 plants can be cut into 2-4 oz. (55-115 g) pieces, which contain lateral buds, and are used as "seed pieces" to produce Greenhouse-2/Field Grown-2 plants. The process of vegetative propagation is repeated to generate planting material.

A.3(e) Evidence of the stability of the genetic changes, including:

(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored

(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments

Because commercial potatoes are vegetatively propagated, the progeny from a parent cell are genetically identical to each other and the parent plant (Section A3(d)). Therefore, the T-DNA insert in Z6 is expected to be genetically stable during vegetative propagation. Consequently, evaluating insert stability by examining inheritance using Mendelian segregation analysis is not applicable for potatoes. Nonetheless, stability of inserted DNA in Z6 was examined across multiple vegetative propagations using Southern blot analysis [unpublished report].

Southern blot analyses were conducted to verify the stability of the two DNA inserts in event Z6 [unpublished report]. Stability of the pSIM1278 and pSIM1678 inserts were assessed in potato plants at different time points.

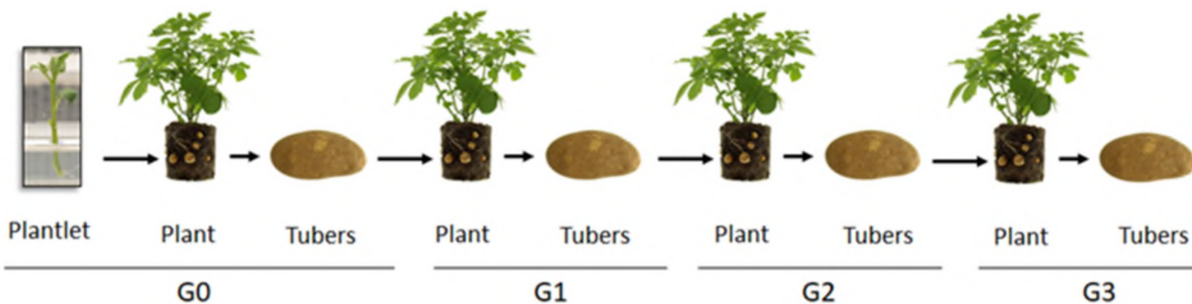
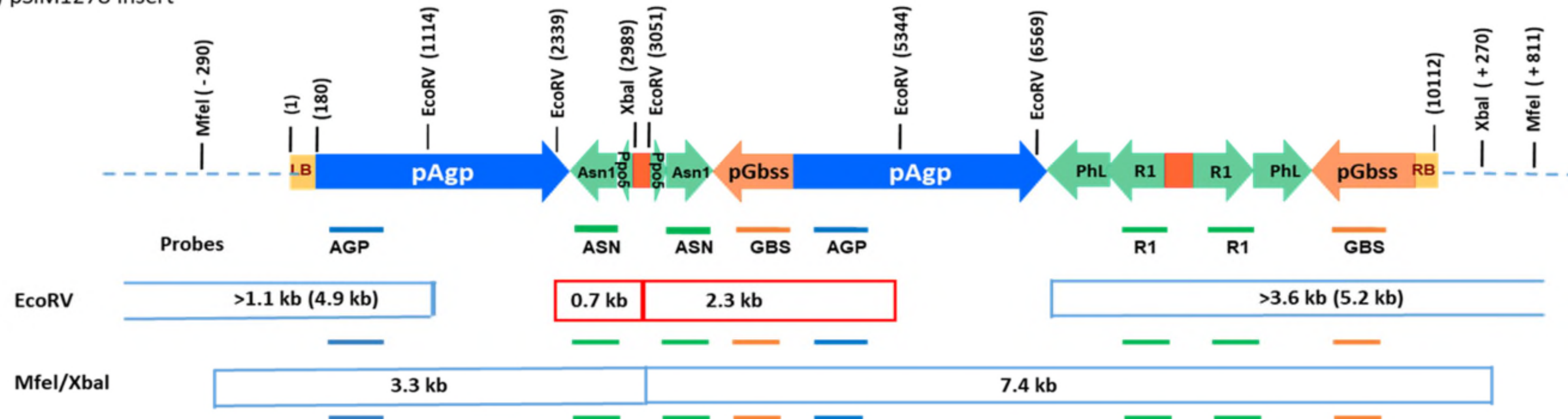


Figure 24. Vegetative Propagation of Potato Plants

Tissue culture plantlets were planted in soil to produce tubers, designated G0. G0 tubers were planted to produce G1 plants and tubers, which were used to produce G2 plants and tubers. DNA was isolated from G0 and G2 samples of vegetatively-propagated greenhouse plants for Southern blot analysis.

<u>**Stability of Event Z6**</u>

The stability of the pSIM1278 and pSIM1678 inserts in Z6 was demonstrated by Southern blot analysis of plants from successive rounds of vegetatively propagated Z6 plants (G0 to G3; Figure 24). Stability was assessed by verifying that the structure of the inserts was consistent with the expected digestion patterns of both inserts and that the banding patterns were consistent between each of the vegetatively propagated plants. Probes from each of the inserts were used to assess the structure following digestion with different restriction enzymes (EcoRV and MfeI/XbaI combination). A schematic diagram of the expected restriction patterns, including fragment sizes and probe specificity, for each digest is provided (Figure 25).



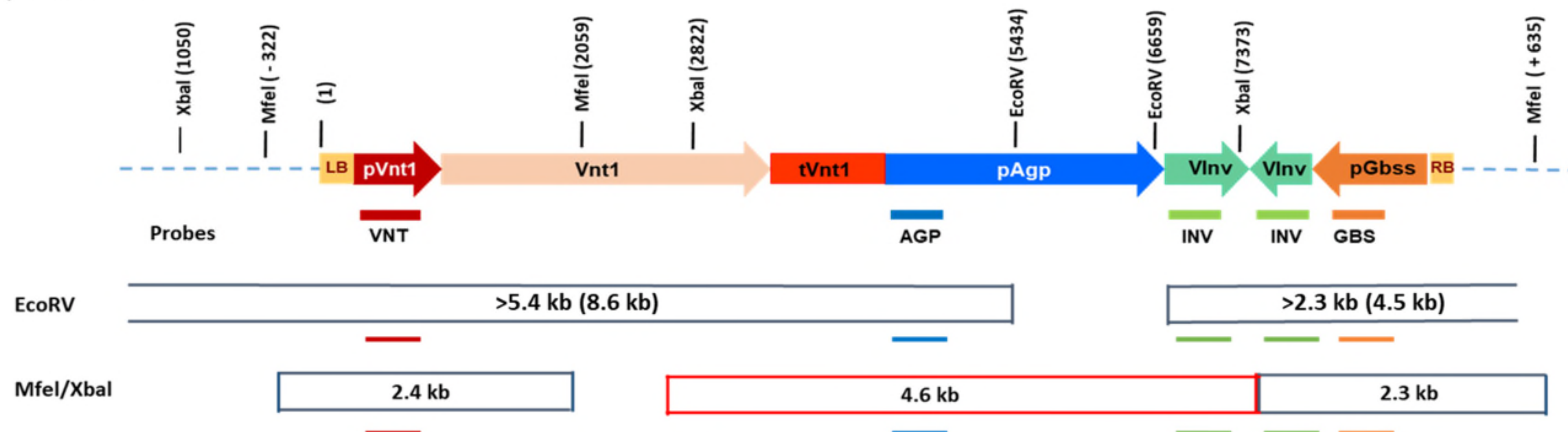


Figure 25. Structure and Expected Digestion Pattern for Z6 Inserts from pSIM1278 and pSIM1678

Structure of (A) pSIM1278 and (B) pSIM1678 inserts in Z6 shown with EcoRV and MfeI/XbaI restriction sites. Digestion products detected by the six probes are indicated below the insert maps. Closed boxes denote fragments fully contained within the insert, whereas open-ended boxes denote fragments extending into the genomic flanking region. A colored bar notes the binding site for the AGP, ASN, GBS, R1, INV, and VNT probes to each digestion product.

The pSIM1278 insert was assessed using four probes AGP, GBS, ASN, and R1 that hybridized to genetic elements of the insert. Similarly, the pSIM1678 insert was assessed using four probes, AGP, GBS, VNT, and INV, that hybridized to elements of the insert. Each probe recognized one or more fragments following digestion with either EcoRV or Mfel/Xbal. The AGP and GBS probes hybridized to fragments associated with both inserts as each contains pAgp and pGbss elements. For clarity, bands associated with each insert are labeled in distinct colors in the accompanying Southern blots (blue, pSIM1278; green, pSIM1678). Bands shared between Snowden and Z6 samples were not labeled as they represent endogenous DNA fragments and are not related to the pSIM1278 or pSIM1678 inserts.

Digestion of the Z6 pSIM1278 insert was expected to produce four fragments (4.9 kb, 0.7 kb, 2.3 kb, and 5.2 kb) after digestion by EcoRV, and two fragments (3.3 kb and 7.4 kb) after digestion by Mfel/Xbal that were detected by the probes. Digestion of the Z6 pSIM1678 insert was expected to produce two fragments (8.6 kb and 4.5 kb) following digestion with EcoRV, and three fragments (2.4 kb, 4.6 kb, and 2.3 kb) when digested by Mfel/Xbal. These fragments were detected by the probes.

Six Southern blots were performed following digestion of Z6 DNA with EcoRV (Figure 26). The banding pattern on each Southern blot was identical for each of the vegetatively propagated Z6 plants (from G0-G3) when hybridized separately with the six probes. The two expected pSIM1278 fragments (4.9 kb and 2.3 kb) and one pSIM1678 fragment (8.6 kb) were observed for each sample when hybridized with AGP. The GBS probe detected two pSIM1278 fragments (2.3 kb and 5.2 kb) and one pSIM1678 fragment (4.5 kb) in each Z6 sample. The ASN and R1 probes are unique to the pSIM1278 insert. The expected fragments (0.7 kb and 2.3 kb) were observed with ASN, while R1 detected the 5.2 kb fragment, which has higher intensity compared to the Snowden band. The pSIM1678-specific probes, INV and VNT, identified the expected 8.6 kb and 4.5 kb fragments, respectively.

A second set of Southern blots was performed following digestion with restriction enzymes, Mfel/Xbal, to increase the rigor of the structural comparison between samples (Figure 27). Again, the same banding pattern was observed for each of the vegetatively propagated plants (from G0-G3) when hybridized with the six probes. The two expected pSIM1278 fragments (3.3 kb and 7.4 kb) and one pSIM1678 fragment (4.6 kb) were observed for each sample when hybridized with AGP. The one expected pSIM1278 fragment (7.4 kb) and the one pSIM1678 fragment (2.3 kb) were detected by the GBS probe. The pSIM1278-specific probes, ASN and R1, detected the expected fragments (3.3 kb and 7.4 kb, ASN; 7.4 kb, R1) in each Z6 sample. The expected 2.4 kb fragment was observed with pSIM1678-specific VNT probe. Two expected pSIM1678 fragments (4.6 kb and 2.3 kb) were also detected by the pSIM1678-specific INV probe.

The same banding pattern was observed in all Southern blots for each of the vegetatively propagated Z6 plants (Figure 26, Figure 27), which corresponded to the expected digestion pattern in all cases (Figure 25).

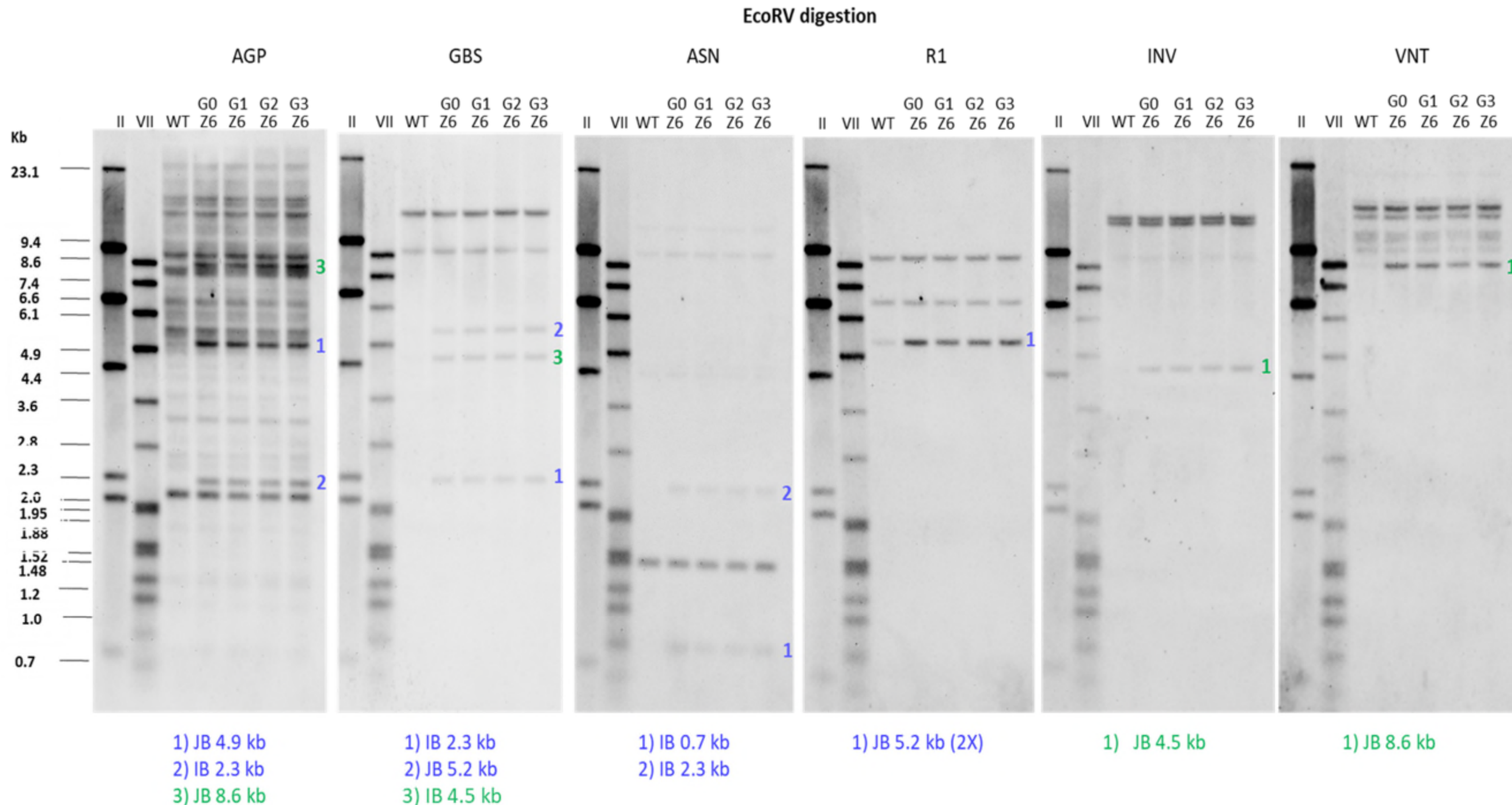


Figure 26. EcoRV Digests: pSIM1278 and pSIM1678 Z6 Inserts are Stable during Vegetative Propagation

Southern blots of Snowden (WT) and Z6 (G0 to G3) genomic DNA following digestion with EcoRV. Southern blots hybridized with AGP, GBS, ASN, R1, INV, or VNT probe. Sizes of insert bands from pSIM1278 (blue) and pSIM1678 (green) are given below the blots. Band sizes correspond to expected products (Figure 25). Molecular weight markers, Dig II (II) and Dig VII (VII), were included in each gel and sizes are labeled to the left of the first gel.

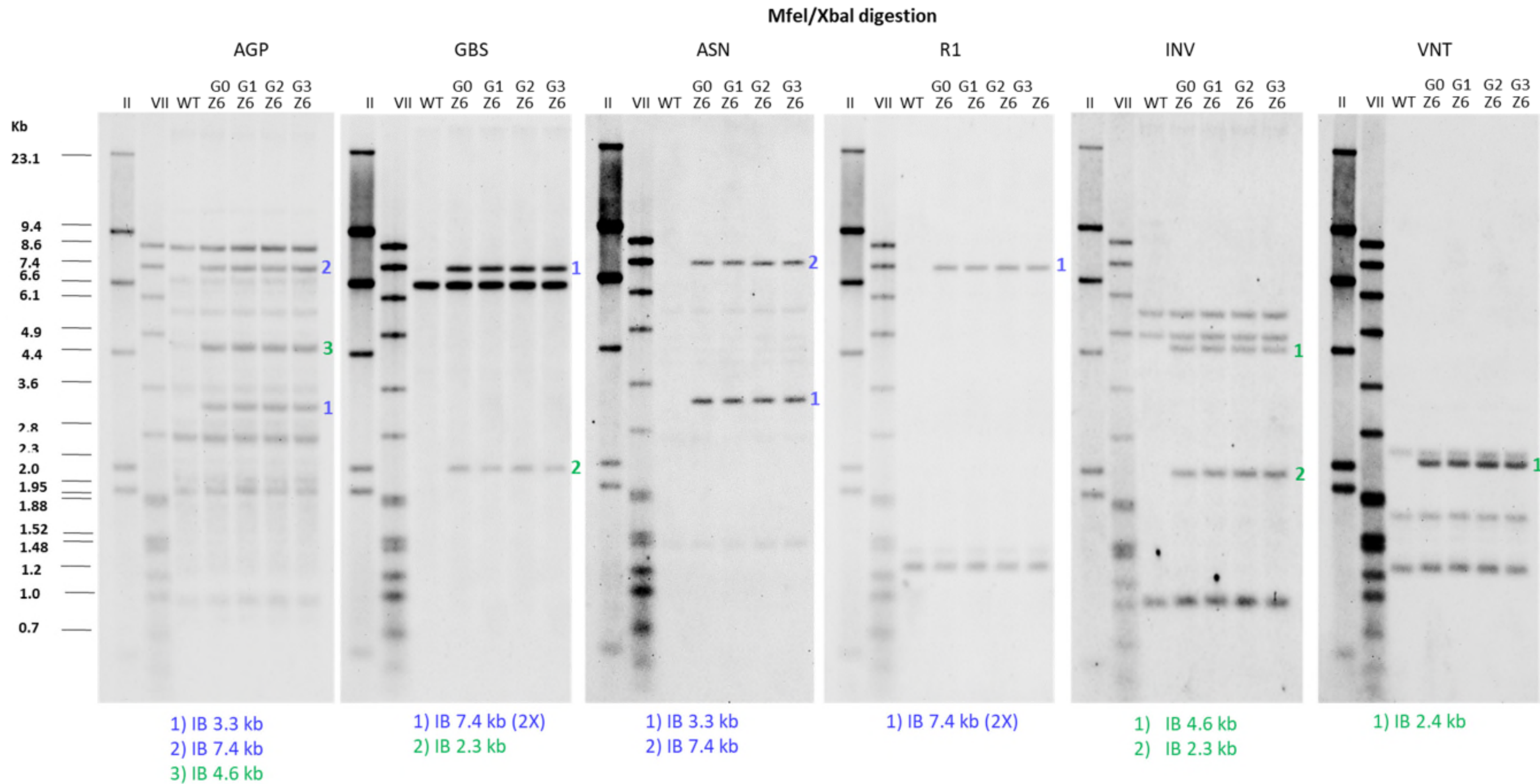


Figure 27. MfeI/XbaI Digests: pSIM1278 and pSIM1678 Z6 Inserts are Stable during Vegetative Propagation

Southern blots of Snowden (WT) and Z6 (G0 to G3) genomic DNA following digestion with MfeI/XbaI. Southern blots hybridized with AGP, GBS, ASN, R1, INV, or VNT probe. Sizes of insert bands from pSIM1278 (blue) and pSIM1678 (green) are given below the blots. Band sizes correspond to expected products (Figure 25). Molecular weight markers, Dig II (II) and Dig VII (VII), were included in each gel and sizes are labeled to the left of the first gel.

Conclusion for Event Z6 Insert Stability

The Southern blot results showed consistent banding patterns with Snowden and Z6 plants, across a number of time points. The consistent banding patterns demonstrated stability of both inserts in Z6. As potatoes are vegetatively propagated, all progeny produced from an individual plant are genetically identical. Consequently, evaluating insert stability by examining inheritance using Mendelian segregation analysis is not applicable for potatoes. However, this study shows that the inserts in Z6 are stable and maintained through vegetative propagation.

A.3(g) An analysis of the expressed RNA transcripts, where RNA interference has been used

In event Z6, reduced black spot, reduced free asparagine, and lower reducing sugars were achieved using RNAi to target five potato RNA transcripts for down regulation (Table 2).

The promoters that drive down regulation are primarily active in tubers. The effectiveness of target gene down regulation and tissue specificity was evaluated by comparing the mRNA levels of the targeted transcripts in tubers, leaves, stems, roots, and flowers in each event using northern blot analysis.

Black spot is a post-harvest physiological phenomenon primarily resulting from the handling of potato tubers during harvest, transport, and processing, and refers to the black or greyish colour that may form in the interior of damaged potatoes. The enzymatic darkening and discolouration, associated with the enzyme polyphenol oxidase (PPO), occurs when PPO leaks out from the plastids of damaged potatoes. Potatoes that show black spot are typically trimmed, or oftentimes the entire potato is rejected before processing. This results in quality control challenges, economic loss, or both. The PPO cassette in pSIM1278 targets polyphenol oxidase transcripts to down regulate enzyme expression via RNAi.

Target Transcript Down Regulation in event Z6

Expression analysis shows reduced expression for the five target transcripts in tubers with expression of the five targeted transcripts in other tissues unaffected. The 18S rRNA levels remained consistent across samples allowing for direct comparisons of asparagine synthetase, polyphenol oxidase, phosphorylase L and water dikinase transcripts between samples in a given tissue. Results are summarised in Table 7.

Table 7. Summary of Down-Regulated Transcripts in Z6 Plants

Transcript	Tuber	Leaf	Stem	Root	Flower
Asparagine synthetase	↓	-	-	-	-
Polyphenol oxidase	↓	-	-	-	-
Phosphorylase L	↓	-	-	-	-
Water dikinase	↓	-	-	-	-
Vacuolar invertase	↓	-	-	-	-

(↓) reduced expression, (-) no change.

Down regulation in Z6 Tubers

RNA was isolated from field-grown Z6 and Snowden control plants (three biological replicates) and analysed by northern blot to determine the extent of down regulation of the target genes. The methods used to assess target gene down regulation are described in [unpublished report].

The 18S RNA and total RNA levels were consistent across samples, allowing direct comparison of Asnl, Ppo5, Phl, Rl, and Vlnv transcripts between samples. The absence or decreased intensity of bands in Z6 tuber samples probed for the target transcripts indicated that Asnl, Ppo5, Phl, Rl, and Vlnv transcripts were down regulated in Z6 tubers (Figure 28). The down regulation of Asnl, Ppo5, Phl, and Rl in Z6 is consistent with the results in V11 [unpublished report], as expected.

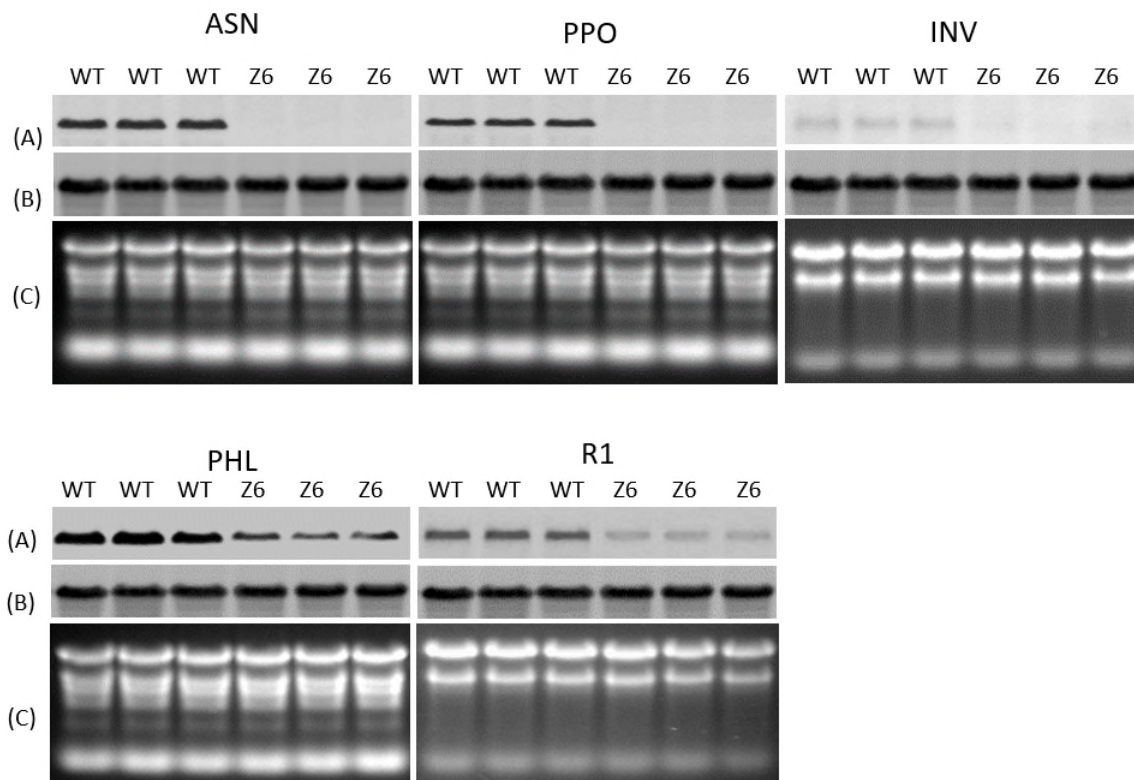


Figure 28. Target Transcript Down Regulation in Z6 Tubers

Northern blots were probed for the RNAi target transcripts: Asn1, Ppo5, PhL, R1 and VInv. (A) Samples analysed with the indicated probe. (B) 18S RNA as a gel loading control. (C) Total RNA is shown stained with ethidium bromide. Three biological replicates were evaluated in each gel.

PPO Activity in Event Z6

The trait efficacy of polyphenol oxidase (PPO) down regulation in potato event Z6 was compared with Snowden [unpublished report]. PPO activity was measured by monitoring the conversion of an exogenous diphenolic substrate, catechol, into melanin which is associated with a visible colour change. An exogenous substrate was used to increase the rate and extent of colour change in the samples.

The PPO activity was compared in fresh and stored tubers from Z6 plants to Snowden (Figure 29). These data indicated that PPO activity was very low in Z6 plants relative to Snowden and that the trait remained stable over the six-month storage time period. Less PPO activity results in less dark spot formation in Z6 tubers compared to conventional Snowden tubers.

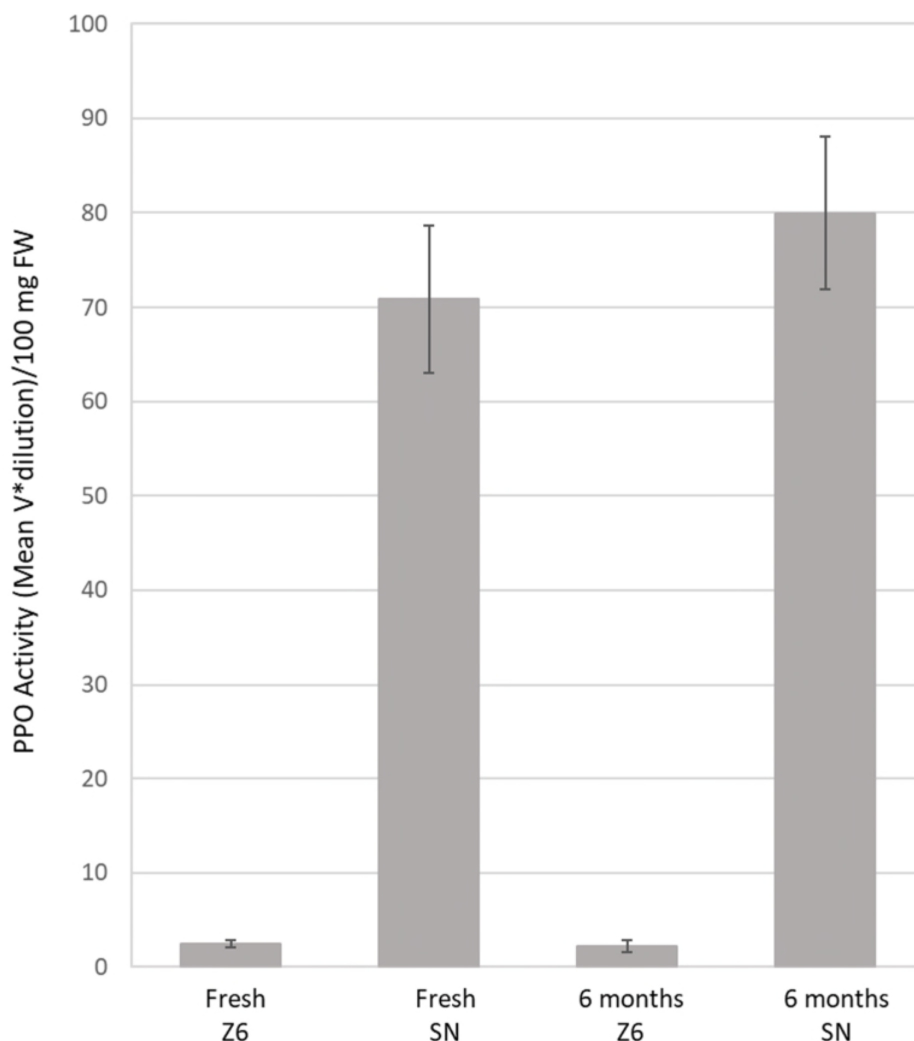


Figure 29. Reduced PPO Activity in Z6 Tubers

PPO activity assay performed on fresh and stored Z6 and Snowden (SN) tubers. Low PPO activity was observed in Z6 compared to the SN conventional tubers. PPO activity was calculated by taking the average of four biological replicates.

Compared to Snowden, PPO activity was reduced in Z6 tubers and remained reduced after six months of storage, which is consistent with effective down regulation of PPO. Decreased PPO activity in Z6 tubers reduces the occurrence of black spot.

Conclusion of the Target Transcript Down Regulation of Event Z6

The *Agp* and *Gbss* promoters are well characterised and are known to be active in tubers and stolons while inducing some transcription in photosynthetically active tissues and roots (Nakata et al., 1994; Visser et al., 1991). Therefore, reduced target mRNA levels mediated by transcription of the inverted repeats were predicted to be strong in tubers, and less pronounced in leaves, stems, and roots. As expected, down regulation of the targeted transcripts was more effective in tubers than in the flowers, roots, stems, or leaves.

The reduced expression of asparagine synthetase, polyphenol oxidase, and vacuolar invertase were consistent with the compositional and trait efficacy data in Sections B.1 and B.5. Although the down regulation of phosphorylase L and water dikinase transcripts was less effective, the intended trait of lower reducing sugars is still prevalent in these events from the down regulation of vacuolar invertase, as shown in the trait efficacy assessment (Section B.5).

Conclusion of the Genetic Characterisation of Z6

A combination of Sanger and Illumina NGS sequencing corroborated studies using ddPCR and Southern blots, showing the presence of single inserts associated with transformation of Z6 using pSIM1278 and pSIM1678. The structure and sequences of the two inserts in Z6 are provided, with flanking DNA sequence. No backbone DNA was integrated into the Snowden genome. No annotated genes were disrupted by the insertion of these T-DNAs.

The inserts in Z6 consisted solely of the sequences targeted for insertion and did not contain any detectable backbone sequence. The studies confirmed the stability of the DNA inserts in Z6 across multiple cycles of vegetative propagation, which demonstrated that inserts likely will be maintained through vegetative propagation.

B. Characterisation and Safety Assessment of New Substances

B.1. Characterisation and Safety Assessment of New Substances

B.1(a) a full description of the biochemical function and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions

Late blight protection was achieved by expression of the VNT1 protein in Z6. Expression was obtained using the same cassette and the same protein that was added added into Events W8, X17 and Y9, which were previously reviewed and approved by FSANZ in A1139. The structure, expression and mode of action of VNT1 are the same in Z6 as in W8, X17, Y9.

VNT1 specifically recognises the *P. infestans* secreted Avr-vnt1 effector, enabling the plant to initiate its immune response (Pel, 2010). The immune response acts through a conserved network of signaling pathways and induces a hypersensitive response (Coll et al., 2011; Panstruga et al., 2009). The hypersensitive response destroys infected plant tissue through programmed cell death, restricting growth and spread of the pathogen.

The pSIM1678 vector includes an expression cassette for the *Rpi-vnt1* gene originating from *Solanum venturii*. The gene product, VNT1, is an R-protein involved in the plant hypersensitive response that protects potato against late blight infection from *P. infestans*.

Expression of the *Rpi-vnt1* mRNA in Z6 is driven by its native promoter and terminator. Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) was used to verify gene expression of the *Rpi-vnt1* transcript in Z6 leaves and tubers. Total RNA was isolated from tissues of Z6, and their respective control varieties and subjected to RT-qPCR using *Rpi-vnt1* specific primers. Gene expression of *Rpi-vnt1* was normalised to a set of endogenous housekeeping genes, α-tubulin, and elongation factor 1α, within each sample.

The expression of VNT1 in events Z6 is summarised with full details presented in the following study:

- [unpublished report], Z6

<u>VNT1 Expression in Event Z6</u>

VNT1 levels were determined by western blot using VNT1 antibodies. A full-length VNT1 protein standard was expressed in *E. coli* and used for VNT1 quantitation and detection purposes. Expression, purification, and quantification of the full-length VNT1 protein standard was described in [unpublished report]. The limit of quantitation (LOQ) of the western blot method was established using samples of Russet Burbank initially transformed with pSIM1678. The VNT1 western blot methods were then applied to other pSIM1678 transformed plants, including Z6 [unpublished report].

<u>Determining Endogenous VNT1 Concentrations using Western Blot</u>

The VNT1 protein standard was used as a positive control to compare endogenous levels of VNT1 in Z6 plants. The VNT1 protein standard was analyzed alone or spiked into Z6 and Snowden lysates at two different concentrations (2.5 ng and 1.25 ng). Proteins extracted from Z6 and Snowden tuber and leaf tissues (10 µg) were mostly consistent in yield and banding patterns, as shown in the stain-free gels (left panels, Figure 30). There were some slight differences observed between the lanes in the leaf samples, which do not appear to impact VNT1 detection. Other bands that are not VNT1 are observed on the blots. These bands are in both the Z6 and SN samples and have different sizes compared to the VNT1 standard (VNT1 STD) (right panels,

Figure 30). These data indicate that VNT1 concentrations in Z6 are below the LOQ of 500 ppb in tuber and leaf.

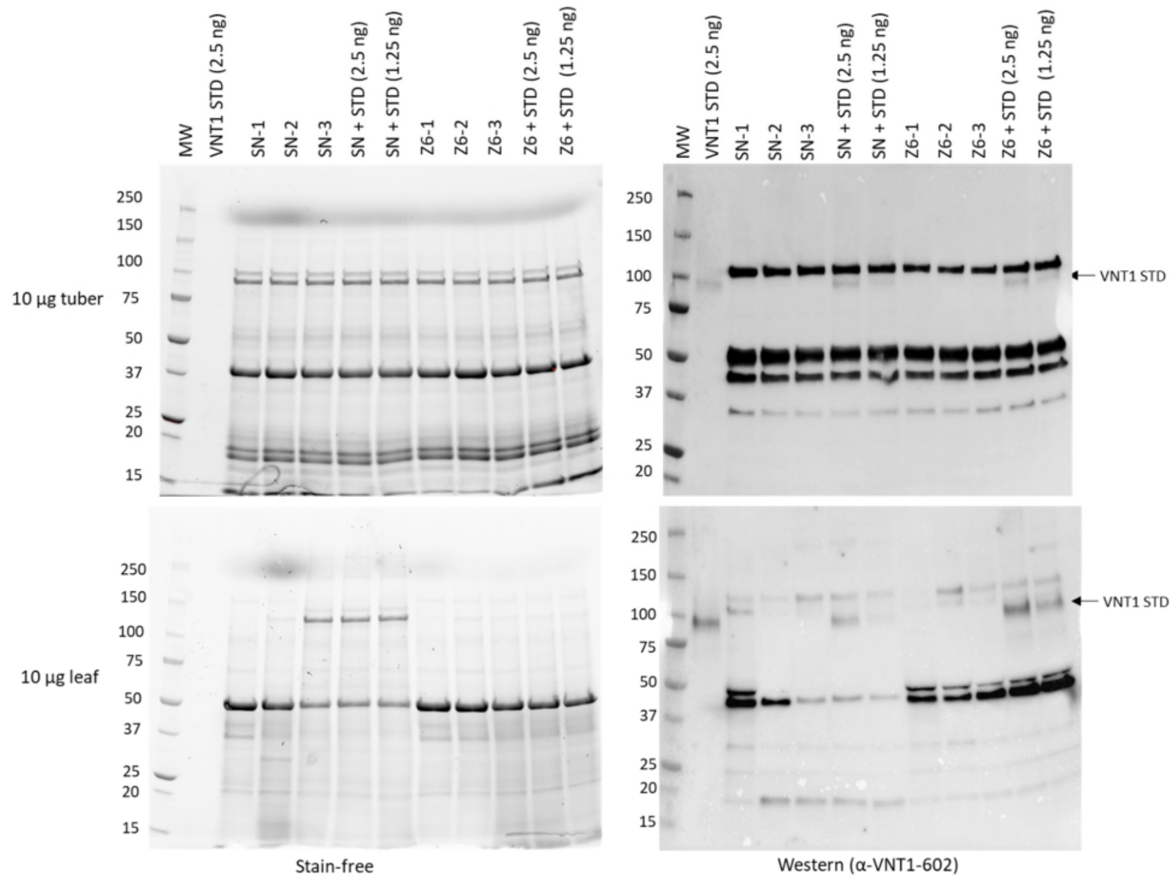


Figure 30. VNT1 was Undetected in Z6 by Western Blot

The VNT1 protein was undetected in three biological replicates of Z6 leaf and tuber tissues following western blot analysis using the α-VNT1-602 antibody. Z6 and Snowden (SN) samples were spiked with 2.5 ng and 1.25 ng of the VNT1 protein standard. Stain free SDS-PAGE gels show the amount of protein loaded in each lane (left). VNT1 was not detected in Z6 or SN tissue samples (right). Tuber is shown in the top panels and leaf in the bottom panels.

Analysis of *Rpi-vnt1* Transcript Expression by RT-qPCR

RT-qPCR was used to measure *Rpi-vnt1* transcript levels in Z6 and Snowden leaf, stem, root, flower, and tuber tissues. Primers and probes were specific to the LRR region of *Rpi-vnt1*. Achieving specificity in the assay was a challenge due to *Rpi-vnt1* homologs present in Snowden, however, amplification was largely limited to Z6 and *S. venturii*, which served as a positive control (Figure 31).

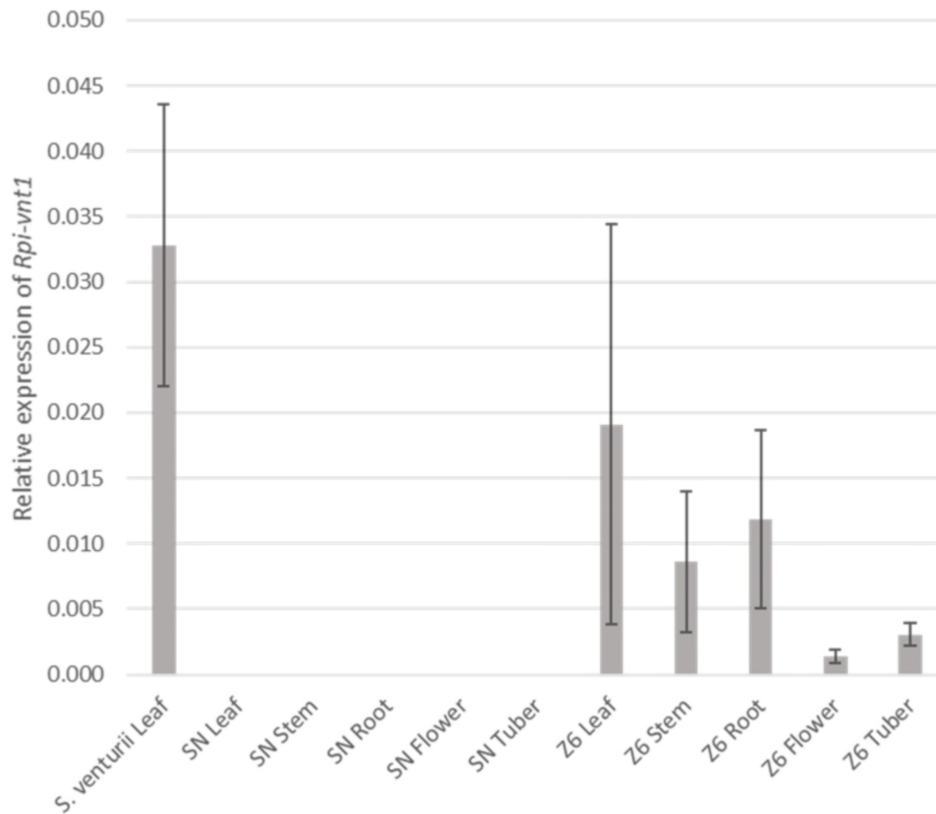


Figure 31. *Rpi-vnt1* Transcript Levels in Z6 Measured by RT-qPCR

The *Rpi-vnt1* RT-qPCR assay was performed on three biological replicates of Z6 and Snowden (SN) leaf, stem, root, flower, and tuber tissues, analysed in triplicate. Expression of *Rpi-vnt1* was normalized to endogenous reference genes (*Elongation Factor 1α* and *APRT)* by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression by $2^{-\Delta Cq}$, showing relative expression of *Rpi-vnt1* for all samples analyzed. *S. venturii* leaf tissue was used as a positive control.

CONCLUSION

The *Rpi-vnt1* gene, which encodes the VNT1 protein and confers resistance to late blight, was introduced into Snowden to generate Z6. A western blot assay, with a VNT1-specific antibody, was used to detect VNT1 in Z6 plants. VNT1 was not detected in Z6 leaf and tuber field samples, and the amount of VNT1 is estimated to be below the LOQ, which was conservatively established to be less than 500 ppb. To show that *Rpi-vnt1* is expressed in Z6, even though VNT1 was not detected in Z6 leaf and tuber tissues, and *Rpi-vnt1* transcript expression analysis was performed using RT-qPCR. The transcript expression results show that *Rpi-vnt1* is expressed in Z6 tissues and suggests that expression levels are low.

B.1(b) Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.

Safety Assessment of the VNT1 Protein

A weight-of-evidence approach using risk assessment principles was used to evaluate the safety of the VNT1 protein. This approach was presented and assessed by FSANZ in application A1139 and considered all data in a comprehensive manner to evaluate the safety of VNT1, including risk assessment results (potential hazard X potential exposure = potential risk).

The weight-of-evidence strongly supports VNT1 safety:

- The *Rpi-vnt1* gene is identical to the *Rpi-phu1* gene found in *S. phureja* potatoes, which has an established history of safe use. Therefore, the introduction of *Rpi-vnt1* into commercially grown potatoes should result in varieties with substantially similar food safety profiles to varieties with *Rpi-phu1*;
- The prevalence of R-genes similar to VNT1 in edible crops suggests that R-proteins are widespread in nature, and the VNT1 protein is similar to proteins already present in the food supply with a history of safe consumption;
- The biological mechanism of disease protection by VNT1 is the triggering of an existing hypersensitivity response in the plant, which is conserved among plants containing R-genes. This supports the safe use of VNT1 to introduce disease protection into edible crops;
- Bioinformatic analysis confirms that VNT1 lacks sequence similarity to known toxins and allergens;
- Homology of VNT1 to other proteins in tomato, pepper, and potato with a history of safe use provides additional evidence that VNT1 in Innate® potatoes is as safe for human consumption as R-proteins similar to VNT1 in other foods; and
- The potential exposure for humans and livestock to VNT1 is negligible.

Based on the weight-of-evidence and taking into account the close-to-zero risk, the VNT1 protein in Innate® potato is as safe as conventional varieties for humans, livestock, and the environment.

B.1(c) information on whether any new protein has undergone any unexpected post-translational modification in the new host

The VNT1 protein is expressed from a potato gene, *Rpi-vnt1* that is native to a related species, *S. venturii*. The cassette that was used to introduce this gene into Russet Burbank, Ranger Russet, and Atlantic varieties, included the native promoter and terminator sequences from the original host, *S. venturii*. The transformed varieties show efficacy against *P. infestans* and expression studies showed similar levels of expression of the gene in foliar tissue when compared to *S. venturii*, suggesting that the gene and protein are processed similarly. Post-translational modifications (PTMs) to VNT1 cannot be evaluated as expression levels are below the limit of detection, but differences in PTMs due to cellular processing enzymes are unlikely given that the original and new hosts are closely related. Thus, differences in PTMs of VNT1 between the hosts are not expected.

B.1(d) where any ORFs have been identified (in subparagraph A.3(c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs

The sequences of the genetic elements in the pSIM1278 and pSIM1678 cassettes are derived from Ranger Russet conventional potatoes with the exception of *Rpi-vnt1*, which is from the wild species *Solanum venturii* (Foster et al., 2009). Expression of allergens or toxins is unlikely, particularly in potatoes transformed with potato DNA.

An analysis was completed using bioinformatic techniques (Goodman et al., 2008; Ladics et al., 2007; Terrat and Ducancel, 2013) to determine homology between known toxins or allergens and open reading frames (ORFs) introduced into event Z6 through transformation with pSIM1278 and pSIM1678 [unpublished report]. A summary of the methods used to identify ORF sequences and evaluate the sequences against known allergens or toxins is provided in Table 8. Most of the ORFs contained in the pSIM1278 and pSIM1678 inserts already exist as part of the potato genome, as the inserts are derived from potato DNA.

Table 8. Overview of Analyses Using Bioinformatics

Analysis	Purpose	Approach
Start-to-stop ORF Analysis	Identify all open reading frames associated with the pSIM1278 and pSIM1678 inserts, including junction regions.	Python script: systematically identify all ORFs (≥30 amino acids) located between a start codon and a stop codon where all six reading frames are considered.
Allergenicity Analysis	Confirm that known allergenic sequences have not been introduced through transformation.	AllergenOnline (FASTA Search): identify any small regions of identity or larger regions of homology between ORFs and known allergens.
Toxicity Analysis	Confirm that sequences similar to known toxins have not been introduced through transformation.	BLAST (blastp) search: identify any ORFs with homology to proteins with “toxin” in its NCBI annotation.

The ORFs identified from the pSIM1278 and pSIM1678 inserts were assessed for homology to potential toxins and known allergens. The sequences associated with each ORF are shown in Appendix A of [unpublished report]. The ORF sequences were separated into three categories:

1. Gene sequences
    - The pSIM1678 insert contains the *Rpi-vnt1* late blight resistance gene, which expresses the VNT1 protein. There are no other protein expressing genes in the inserts from pSIM1278 or pSIM1678.
2. Junction sequences
    - These include ORFs spanning the insert and flanking genomic DNA.
    - A single ORF was identified at the left junction of each insert. There were no ORFs associated with the right junction from either insert.
3. Insert sequences
    - Most of these ORFs are contained within the individual elements in the inserts and are comprised of potato DNA.

Identification of ORFs Associated with pSIM1278 and pSIM1678

An ORF was defined as a contiguous, ≥30 amino acid sequence between start- and subsequent in-frame stop-codons. Nucleotide sequences were translated in three reading frames from two directions. All six reading frames within the pSIM1278 and pSIM1678 inserts and flanking regions were analyzed for ORFs (shown schematically in Figure 32). The results were converted into FASTA-formatted files using CLC Genomic Workbench software (Qiagen). An ORF was considered novel if it would not otherwise exist in the potato genome.

All ORFs were identified that were contained within the Z6 inserts, including those adjacent to flanking genomic sequence (junction ORFs). Duplicate ORFs that resulted from having repetitive elements in the insert (e.g. pAgp and pGbss) were removed.

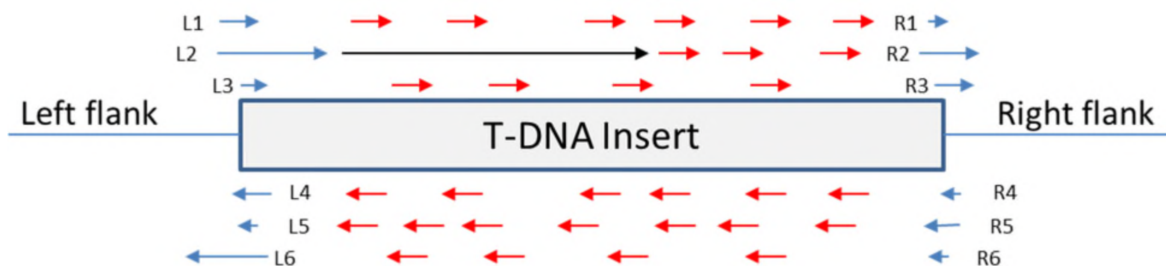


Figure 32. Complete ORF Analysis Scheme

A representative T-DNA insertion site in the plant genome. All ORFs ≥30 amino acids, contained within the DNA insert (red lines), including an introduced protein coding region (**black** line) or overlapping the junctions between the insert and plant genome (blue lines), were identified and used in subsequent analyses. All lines are representative and do not indicate actual ORFs.

Allergenicity Searches

Searches were performed to identify homology of ORF sequences, from the pSIM1278 and pSIM1678 inserts in Z6, to known allergens in the AllergenOnline.org database. AllergenOnline is made available by the Food

Allergy Research and Resource Program (FARRP) at the University of Nebraska (http://www.allergenonline.org/databasefasta.shtml). Sequences deposited in the database are reviewed annually by a panel of qualified food allergenicity experts. Version 19 of the AllergenOnline database (available on February 10, 2019) contains 2,129 sequences, and was searched using FASTA to identify sequence identity between input and database entries (Pearson and Lipman, 1988). The search methods used are supported by recent published guidance for protein allergenicity prediction in food products, and are based on sequence identity as well as structural aspects of the interaction between antibodies and protein targets (cross-reactivity potential) (Goodman et al., 2008; Ladics, 2008; Ladics and Selgrade, 2009).

Gene Sequences

No homologous allergens were identified for the coding sequence of *Rpi-vnt1* (VNT1), using the full-length, 80-mer, or 8-mer searches.

Junction Sequences

No homologous allergens were identified for any ORFs associated with junctions between inserts and genomic DNA using the full-length, 80-mer, or 8-mer searches.

Insert Sequences

Two ORFs associated with the VInv sequence from the pSIM1678 insert matched with a minor allergen, beta-fructofuranosidase precursor-vacuolar invertase, from tomato (Table 9). The ORFs are complementary as a result of the VInv inverted repeat and have similar sequences (Table 9, bold sequence). This similarity results in a common match being identified from the bioinformatics analysis.

Table 9. Allergen Homology of pSIM1678 ORFs in Z6

ORF[1]	Query Match	Organism	Accession
>ORF35 **MLSWQRTAYHFQPQKNWMNDPNGPLYHKGWYHL** **FYQYNPDSAIWGNITWGHAVSKDLIHWLYLPFAMVP** **DQWYDINGVWTGSA**TILPDGQIMMLYTGDTDDYVQ VQNLAYPTNLSDPLLLDWVKYKGNPVLVPPPGIGVKDF RGIQRTQSRHR >ORF54 **MLSWQRTAYHFQPQKNWMNDPNGPLYHKGWYHL** **FYQYNPDSAIWGNITWGHAVSKDLIHWLYLPFAMVP** **DQWYDINGVWTGSA**EFL	Minor allergen beta-fructofuranosidase precursor–vacuolar invertase (Foetisch et al., 2003)	*Solanum lycopersicum*	AAL75449, AAL75450

[1]Bold sequence indicates region of identity between ORFs

Although homology was identified between these ORFs and the tomato allergen, it is not a safety concern. Because the ORF sequences exist naturally in potato as part of the vacuolar invertase protein, Z6 potatoes are no more likely than conventional potatoes to cause an allergic reaction in individuals sensitive to the tomato vacuolar invertase. Additionally, translation of the ORF into protein is unlikely because the transcript from the inverted repeat forms a dsRNA that gets processed by the RNAi machinery into small-interfering RNA (siRNA) to prevent ribosomal translation through RNA interference. The VInv cassette leads to

production of siRNA that down-regulate the potato vacuolar invertase gene resulting in reduced amounts of vacuolar invertase in Z6 compared to conventional potatoes and eliminating any allergenicity concerns that may be associated with these ORFs.

Toxicity Searches

Methods familiar to regulators and established in some countries include an approach modeled after the allergenicity studies where bioinformatics is used to inform on the potential of sequence similarity between protein sequences and known toxins.

The NCBI database was queried on March 7, 2019 using all protein sequences annotated with the keyword "toxin" (Entrez query: "toxin"; E-value $< 10^{-2}$). All matches are reported, although many proteins that are not actual toxins may be identified using this search criterion. Matches may include actual toxins, proteins involved in the synthesis of toxins in a host, proteins that interact with toxins, proteins involved in toxin-induced defense responses, or non-toxic proteins from organisms that produce other known toxins. Only proteins that match to known toxins indicate a potential safety concern. For all others, a brief explanation and rationale for safety is included.

ORFs were assessed for homology to toxins by alignment with proteins in the NCBI database limited to accession records annotated with the Entrez keyword, "toxin".

Gene Sequences

No homologous toxins were identified from the coding sequence for VNT1. Some proteins that align with VNT1 were identified because they contain the keyword "toxin" in their accession records. These proteins function to protect plants against toxins or toxin-producing pathogens (Table 10).

LOV1, RP3-like, and Tsn1 are R-protein homologs that function in the sensitivity of their host to fungal pathogens through recognition of effector molecules, i.e. victorin, Pc toxin, and ToxA. A literature review confirmed that these R-proteins are in fact not toxins or substances with toxic properties (Faris et al., 2010; Lorang et al., 2007; Nagy and Bennetzen, 2008; Walton, 1996).

RGA2-like is a predicted disease resistance protein from apple (*Malus domestica*). The alignment between RGA2-like and VNT1 is shown in Figure 33. 2. A 65 amino acid region annotated as BrnT toxin is highlighted in yellow. The sequence similarity between VNT1 and RGA2-like in this region is low, so VNT1 would not be considered homologous to the region annotated as BrnT toxin.

Additionally, this region of the RGA2-like protein appears to have been incorrectly annotated as a toxin. The annotation was made by a predictive software algorithm (Gnomon) in June of 2016, which has not been confirmed by human QC. A BLAST query of the sequence against the NCBI protein database identified over 200 matches (E-value ≤ 1) to eukaryotic proteins, none of which were annotated as toxins or the BrnT toxin. The same BLAST search did not identify this region in prokaryotes.

Table 10. Summary of Toxin Homology for VNT1

ORF	Query Match	Organism	Accession
>ORF32 (VNT1) MNYCVYKTWAVDSYFPFLILTFRKKKFNEKLKEMAEI LLTAVINKSIEIAGNVLFQEGTRLYWLKEDIDWLQRE MRHIRSYVDNAKAKEVGGDSRVKNLLKDIQQLAGD VEDLLDEFLPKIQQSNKFICCLKTVSFADEFAMEIEKI KRRVADIDRVRTTYSITDTSNNNDDCIPLDRRRLFLH ADETEVIGLEDDFNTLQAKLLDHDLPYGVVSIVGMP GLGKTTLAKKLYRHVCHQFECSGLVYVSQQPRAGEI LHDIAKQVGLTEEERKENLENNLRSLLKIKRYVILLDDI WDVEIWDDLKLVLPECDSKIGSRIIITSRNSNVGRYIG GDFSIHVLQPLDSEKSFELFTKKIFNFVNDNWANASP DLVNIGRCIVERCGGIPLAIVVTAGMLRARGRTEHA WNRVLESMAHKIQDGCGKVLALSYNDLPIALRPCFL YFGLYPEDHEIRAFDLTNMWIAEKLIVVNTGNGREA ESLADDVLNDLVSRNLIQVAKRTYDGRISSCRIHDLL HSLCVDLAKESNFFHTEHNAFGDPSNVARVRRITFY SDDNAMNEFFHLNPKPMKLRSLFCFTKDRCIFSQM AHLNFKLLQVLVVVMSQKGYQHVTFPKKIGNMSCL RYVRLEGAIRVKLPNSIVKLKCLETLDIFHSSSKLPFGV WESKILRHLCYTEECYCVSFASPFCRIMPPNNLQTL MWVDDKFCEPRLLHRLINLRTLCIMDVSGSTIKILSA LSPVPRALEVLKLRFFKNTSEQINLSSHPNIVELGLVG FSAMLLNIEAFPPNLVKLNLVGLMVDGHLLAVLKKL PKLRILILLWCRHDAEKMDLSGDSFPQLEVLYIEDAQ GLSEVTCMDDMSMPKLKKLFLVQGPNISPISLRVSE RLAKLRISQVL	LOV1: Confers susceptibility to the fungus *Cochliobolus victoriae* by conditioning victorin-dependent induction of defense-associated proteins. Victorin is a toxin synthesized by *C. victoriae.*	*Arabidopsis thaliana*	A7XGN8, A9QGV6
	RP3-like: Confers resistance to Pc toxin.	*Sorghum bicolor*	ACE86400, ACE86402, ACE86396
	Tsn1: Confers sensitivity to the wheat fungal pathogen ToxA.	*Aegilops speltoides*	ADG84875, ADG84876
	PREDICTED—RGA2-like: Region 107-171 annotated as "Ribonuclease toxin, BrnT, of type II toxin-antitoxin system".	*Malus domestica*	XP_008342325

PREDICTED: disease resistance protein RGA2-like [Malus domestica]

Sequence ID: **XP_008342325.1** Length: 353 Number of Matches: 1

Range 1: 57 to 349

Score	Expect	Method	Identities	Positives	Gaps	Frame
81.6 bits(200)	3e-15()	Compositional matrix adjust.	90/314(29%)	146/314(46%)	39/314(12%)	

Features:

```
Query  93   DSRVKNLLKDIQQLAGDVEDLLDEFLPKIQQSNKFICCLKTVSFADEFAMEIEKIKRRVA  152
            D  + + L  ++ ++ D++D+LDEF  + Q+     +  L T S          + IK +V
Sbjct  57   DHLLTDWLGKLKDVSYDIDDVLDEF--EFQKLRMQVLGLGTGS---------DTIKGKV-  104

Query  153  DIDRVRTTYSITDTSNNNDDCIPLDRRRLFLHADETEVIGLEDDFNTLQAKLLD--HDLP  210
             I    +   +++ + S +            F+HA    +VIG E +    +   +L     H  P
Sbjct  105  -IAAAKAQFNLAERSVDWHGMHMERETHSFVHA--PDVIGRESEKEEIVVQLFKDTHTGP  161

Query  211  -----YGVVSIVGMPGLGKTTLAKKLY--RHVCHQFECSGLVYVSQQPRAGEILHDIAKQ  263
                 V+SI G+ GLGKTTLAK +Y     V    FE     V VS     +   +L +I
Sbjct  162  GDEENVSVISINGLGGLGKTTLAKLVYNDNRVVTNFELRIWVCVSDDFDSKRLLSEIVTA  221

Query  264  VGLTE--EERKENLENNLRSLLKIKRYVILLDDIWD-------VEIWDDLKLVLPECDSK  314
                 +   +E   E ++   LR   L   K+ +++LDD+WD         V+ W DLK +L     +
Sbjct  222  ATSQKCGDESIEQMQMKLRRALTGKKLLLVLDDVWDKGPMGITVKKWIDLKSLLNV--AA  279

Query  315  IGSRIIITSRNSNVGRYIGGDFSIHVLQPLDSEKSFELFTKKIFNFVNDNWANASPDLVN  374
             G +II+T+RN +V    + GD   +H+L+ L        +F  K   F       +        P+L+
Sbjct  280  CGRKIIVTTRNESVALLM-GDAHMHLLKVLPLSDCMTIFVKVAFARREE---QNHPNLMK  335

Query  375  IGRCIVERCGGIPL  388
            IG  IV+   G PL
Sbjct  336  IGEDIVKSVEGFPL  349
```

Figure 33. Alignment of VNT1 with Disease Resistance Protein, RGA2-like, from Apple

The BLAST (blastp) alignment of the VNT1 (Query) with the RGA2-like protein (Sbjct) from apple, which has a region annotated as "BrnT toxin" (highlighted in yellow). The identity between the two proteins is low across the highlighted region.

Junction Sequences

No homologous toxins were identified for any junction ORFs aligned to the entries in the NCBI database.

Insert Sequences

No homologous toxins were identified for ORFs associated with the pSIM1278 or pSIM1678 inserts, when aligned to the entries in the NCBI database.

Alignment of pSIM1278 ORFs with the NCBI protein database identified asparagine synthetase homologs that contain the keyword, "toxin", in their accession record (Table 11). Asparagine synthetase homologs are expressed in many organisms but are not toxins. The pSIM1278 insert contains an inverted repeat containing sequence from the potato asparagine synthetase (*Asn1*) gene. Each side of the inverted repeat produces a unique ORF. The two ORFs are similar in sequence resulting in a common set of protein matches when assessed using bioinformatics (Table 11, bold sequence).

Table 11. Toxin Search Results for pSIM1278 ORFs

<table>
<tr><th>ORF[1]</th><th>Query Match</th><th>Organism</th><th>Accession</th></tr>
<tr><td rowspan="5">>ORF4
MFLMSRKIKSLG
VKMVISGEGADE
IFGGYLYFHKAPN
KEEFHTETCRKIK
ALHQYDCLRANK
ATSAWGLEARVP
FLDKEFEFVMCG
LQKGESTKLQMN
KNKTEIDF

>ORF29
MFLMSRKIKSLG
VKMVISGEGADE
IFGGYLYFHKAPN
KEEFHTETCRKIK
ALHQYDCLRANK
ATSAWGLEARVP
FLDKEFVNKLVIN</td><td>Asparagine Synthetase B or Asparagine Synthase</td><td>Escherichia coli</td><td>AIZ81697, KFD76705, NP_308731, EIL13740, EYZ18706, EYY50888, EZH10307, EIL02389, EYY57333, EIL08426, KDV14966, EYU77209, EIL30960, EZA17347, EYV14508, KNZ12093, EYW20264, EZA37061, EZB27777, EYV89799, EYZ96359, EJF05567, ANO76830, PJI61967, OTB64025, KYR19924, KYT66274, KYU11696, PBQ68604, OTB49553, KYS77729, KYR79236, KYU23155, KYR36119, PAU22677, PBR97907, KYR73496, OTB69004, PBR58416, KYR16525</td></tr>
<tr><td>Asparagine Synthetase B</td><td>Enterobacter cloacae</td><td>ASQ17067</td></tr>
<tr><td>Asparagine Synthetase B</td><td>Shigella flexneri, Shigella boydii, Shigella dysenteriae</td><td>PQO16610, PQM97814, PQM84983, PQN36548, PQN35957, PQN20224</td></tr>
<tr><td>Asparagine Synthetase</td><td>Vibrio cholerae</td><td>EEY43083</td></tr>
<tr><td>Asparagine Synthetase or Asparagine Synthase</td><td>Clostridium botulinum</td><td>KIL09560</td></tr>
</table>

[1]Bold is used to highlight regions of identity between the two ORFs

The alignment of pSIM1678 ORFs with the NCBI protein database identified sucrose-degrading enzymes that are homologs of potato vacuolar invertase and ubiquitously expressed in bacteria, including both pathogenic and non-pathogenic strains (Table 12). The accession records for these proteins contained the keyword "toxin" due to the pathogenicity of the host organism, which is not related to the sucrose-degrading enzymes. The pSIM1678 insert contains an inverted repeat containing sequence from the potato vacuolar invertase gene. Each side of the inverted repeat produces a unique ORF. The two ORFs are similar (Table 12, bold sequence) resulting in a common set of protein matches when assessed using bioinformatics.

Table 12. Toxin Search Results for pSIM1678 ORFs

ORF[1]	Query Match	Organism	Accession
>ORF35 **MLSWQRT** **AYHFQPQK** **NWMNDP** **NGPLYHKG** **WYHLFYQY** **NPDSAIWG** **NITWGHAV** **SKDLIHWLY** **LPFAMVPD** **QWYDING** **VWTGSA**TI LPDGQIMM LYTGDTDDY VQVQNLAY PTNLSDPLL LDWVKYKG NPVLVPPP GIGVKDFRG IQRTQSRHR	Glycosyl Hydrolase Family 32, Sucrose-6-Phosphate Hydrolase	*Escherichia coli*	KYU57217, KYU29761, KYU30531, KOZ65786, NP_311270, EYZ93717, EYV91737, KOZ09537, EZB27384, EYZ68862, EDV64124, EYY18841, EYZ04892, KDV15364, KFD75665, EZQ28926, EYV93762, PBQ65362, PBQ55252, PBQ65657, KYR67720, KYR68655, KYR82912, KYR32265, KYR67990, KYR41985, OTB64516, OTB37363, OTB74045, OTB58160, OTB47925, OTB40657, OTB85828, EYW80343, OTE04890, PBR16851, PBR55824, KYS71623, KYS89136, KYS68613, KYS70798, OTC48590, OTC43741, OTD44623, OTD31325, ARA06893, PAU30426, PAU23617, PJI56720
	Glycosyl Hydrolase Family 32	*Shigella dysenteriae, Shigella flexneri*	PQN16446, PQM92544
	Sucrose-6-Phosphate Hydrolase	*Enterobacter cloacae*	ASQ18782, ASQ18131
>ORF54 **MLSWQRT** **AYHFQPQK** **NWMNDP** **NGPLYHKG** **WYHLFYQY** **NPDSAIWG** **NITWGHAV** **SKDLIHWLY** **LPFAMVPD** **QWYDING** **VWTGSA**EF L	Sucrose-6-Phosphate Hydrolase, Beta-Fructofuranosidase	*Enterococcus faecalis*	EFK77719, ELA02140
	Glycosyl Hydrolase Family 32, Sucrose-6-Phosphate Hydrolase, Levanase, Beta-Fructofuranosidase	*Bacillus cereus, Bacillus anthracis, Bacillus amyloliquefaciens*	BAL16531, EDX61098, PDO99061, AWM53161, AWM45605, AWM49398, AWM45947, AWM49739, AWM53492, AWM53917, AWM46166, AWM49952
	Sucrose-6-Phosphate Hydrolase	*Vibrio cholera*	EEY52113, EEY41740, EEY48061
	Sucrose-6-Phosphate Hydrolase	*Enterococcus faecium*	EFF60448, EFF59908, EFF60441
	Glycosyl Hydrolase Family 32	*Clostridioides difficile*	OYO90556
	Sucrose-6-Phosphate Hydrolase	*Clostridium botulinum, Clostridium butyricum*	KIL08234, APF21264, APF21879
	Sucrose-6-Phosphate Hydrolase	*Staphylococcus hyicus*	AJC95603
	Sucrose-6-Phosphate Hydrolase	*Staphylococcus aureus*	KPE20402, KPE18748
	Hypothetical protein	*Ensifer aridi*	WP_026617525

Alignments of ORFs containing sequence from the potato asparagine synthetase and vacuolar invertase genes did identify non-toxic proteins whose annotation includes the keyword "toxin". Both families of proteins are broadly expressed in pathogenic and non-pathogenic organisms but are not related to the toxicity of those organisms. Accession records for proteins are often annotated with the keyword "toxin" as a result of the source organism's toxicity. The search criteria used (see [unpublished report]) can potentially result in identification of false-positives, as seen here.

CONCLUSION

This study used bioinformatics to assess the allergen and toxin potential of ORFs associated with the pSIM1278 and pSIM1678 inserts in Z6. The ORFs associated with the pSIM1278 and pSIM1678 inserts are unlikely to be translated into protein. Transcription of the inverted repeats within the inserts is driven by opposing promoters (pAgp and pGbss), which limits transcriptional read through. Transcription initiation is expected to be limited to the promoter elements contained within the insert, which except for the pVNT1 element, are not designed for expressing protein. The inverted repeats are designed to produce dsRNA that are processed into siRNA, which are not recognized or translated by the ribosome. The siRNA associated with the PHL/R1 and VInv inverted repeats would prevent accumulation of transcripts due to RNA interference mechanisms. In addition, any unexpected expression would require productive transcription, including capping, splicing, and polyadenylation of messenger RNA (mRNA), nuclear export, and translation into stable protein. Thus, only the pVNT1 element is anticipated to lead to production of a protein, i.e. VNT1. As was shown above, based on bioinformatics, none of the ORFs associated with the inserts in Z6 are homologous to known toxins or allergens.

Homology between the pSIM1278 and pSIM1678 insert-associated ORFs and known allergens was limited to an expected minor allergen in tomatoes, whose potato homolog is targeted for reduced expression in Z6. The presence of a potato vacuolar invertase sequence within the pSIM1678 inverted repeat led to ORFs with homology to a minor tomato allergen. As the *VInv* gene and sequences comprising the VInv inverted repeat are found in conventional potatoes, Z6 potatoes are no more likely than conventional potatoes to cause an allergic reaction in individuals sensitive to the tomato vacuolar invertase protein. The VInv cassette produces dsRNA that reduce expression of invertase in Z6, reducing levels of the potato protein homologous to the minor tomato allergen.

No toxins were identified with homology to the ORFs associated with the inserts in Z6. Proteins with homology to VNT1 were not toxins, but plant disease resistance proteins that protect against toxins and toxin-producing pathogens. Asparagine synthetase and sucrose degrading enzymes identified as homologs to ORFs associated with the asparagine synthetase and vacuolar invertase inverted repeats in Z6 potatoes are expressed ubiquitously in bacteria and are not toxins.

B.2. New Proteins

B.2 (a) and (b) Information on potential toxicity and allergenicity

The pSIM1678 T-DNA contains the *Rpi-vnt1* gene found in *S. venturii* and *S. phureja.* The gene is expressed under the native *Rpi-vnt1* promoter and terminator. A detailed description of the history and mode of action of VNT1 can be found in Section B.1(b).

Details of the potential toxicity and allergenicity of the protein VNT1 are presented in the following Sections:

- Section A.2(a)(i) and
- Section B.1(d)

<u>VNT1 Protein Identity</u>

The *Rpi-vnt1* gene (accession: FJ423044) is one of three isoforms identified in the wild species *S. venturii*. The *Rpi-vnt1* gene sequence is identical to the *Rpi-phu1* gene from the related species, *S. phureja,* and a homolog of the Tm-2[2] tomato mosaic virus (ToMV) disease resistance gene in tomato (Foster et al., 2009; Śliwka et al., 2013). The gene encodes the 891 amino acid R-protein designated VNT1 (Figure 34).

The recognition of pathogen-secreted effectors (e.g. Avr-vnt1) by R-proteins is one of the most studied mechanisms in plant defense response (Panstruga et al., 2009). R-proteins such as VNT1 are signal transduction ATPases with homologs found in all domains of life (Leipe et al., 2004). Most known disease resistance R-proteins contain a nucleotide-binding site (NBS or NB) and leucine-rich repeat (LRR) domain (Lozano et al., 2012). Two classes of R-proteins with distinct motifs have been identified that contain the NBS/NB-LRR domains:

- N-terminal toll/interleukin 1 receptor (TIR)(TIR-NB-LRR)
- N-terminal coiled-coil (CC-NB-LRR).

The *Rpi-Vnt1* gene and other R-genes that provide protection against *P. infestans* (known as *Rpi* genes) typically encode immune receptor proteins of the coiled coil, nucleotide binding, leucine rich repeat (CC-NB-LRR) class of intracellular plant proteins (Vleeshouwers et al., 2011).

```
001 MNYCVYKTWA VDSYFPFLIL TFRKKKFNEK LKEMAEILLT AVINKSIEIA
051 GNVLFQEGTR LYWLKEDIDW LQREMRHIRS YVDNAKAKEV GGDSRVKNLL
101 KDIQQLAGDV EDLLDEFLPK IQQSNKFICC LKTVSFADEF AMEIEKIKRR
151 VADIDRVRTT YSITDTSNNN DDCIPLDRRR LFLHADETEV IGLEDDFNTL
201 QAKLLDHDLP YGVVSIVGMP GLGKTTLAKK LYRHVCHQFE CSGLVYVSQQ
251 PRAGEILHDI AKQVGLTEEE RKENLENNLR SLLKIKRYVI LLDDIWDVEI
301 WDDLKLVLPE CDSKIGSRII ITSRNSNVGR YIGGDFSIHV LQPLDSEKSF
351 ELFTKKIFNF VNDNWANASP DLVNIGRCIV ERCGGIPLAI VVTAGMLRAR
401 GRTEHAWNRV LESMAHKIQD GCGKVLALSY NDLPIALRPC FLYFGLYPED
451 HEIRAFDLTN MWIAEKLIVV NTGNGREAES LADDVLNDLV SRNLIQVAKR
501 TYDGRISSCR IHDLLHSLCV DLAKESNFFH TEHNAFGDPS NVARVRRITF
551 YSDDNAMNEF FHLNPKPMKL RSLFCFTKDR CIFSQMAHLN FKLLQVLVVV
601 MSQKGYQHVT FPKKIGNMSC LRYVRLEGAI RVKLPNSIVK LKCLETLDIF
651 HSSSKLPFGV WESKILRHLC YTEECYCVSF ASPFCRIMPP NNLQTLMWVD
701 DKFCEPRLLH RLINLRTLCI MDVSGSTIKI LSALSPVPRA LEVLKLRFFK
751 NTSEQINLSS HPNIVELGLV GFSAMLLNIE AFPPNLVKLN LVGLMVDGHL
801 LAVLKKLPKL RILILLWCRH DAEKMDLSGD SFPQLEVLYI EDAQGLSEVT
851 CMDDMSMPKL KKLFLVQGPN ISPISLRVSE RLAKLRISQV L
```

Figure 34. Amino Acid Sequence of VNT1

The *Rpi-vnt1* gene encodes an 891 amino acid R-protein (102 Kda). Individual domains are highlighted, coiled-coil (CC) (green), nucleotide binding (NBS) (blue), and leucine rich repeat (LRR) (brown).

B.3. Other (non-protein) new substances

If other (non-protein) substances are produced as a result of the introduced DNA, information must be provided on the following:

B.3(a) the identity and biological function of the substance

B.3(b) whether the substance has previously been safely consumed in food

B.3(c) potential dietary exposure to the substance

Transcription of the inverted repeats leads to down regulation of asparagine synthetase, polyphenol oxidase, phosphorylase L, water dikinase, and vacuolar invertase through production of dsRNA and the plant's RNAi pathway. The inverted repeats are derived from the DNA sequences from five potato genes (*Asn1*, *Ppo5*, *PhL*, *R1,* and *VInv*; Table 2).

The T-DNA in each plasmid contain down-regulation cassettes, that result in the production of siRNA in Z6 using the plant's RNAi pathway. As described in Section A.3(b), each down-regulation cassette is comprised of DNA sequence arranged as an inverted repeat.

Due to the nature of the inverted repeat sequences, their transcripts form dsRNA through complementary binding. The dsRNA act as a precursor for the plant's own RNAi post-transcriptional regulatory pathway. A cellular RNase III enzyme, Dicer, recognises and processes the precursor dsRNA into 21-24 bp duplexes termed siRNA. The siRNA bind with cellular proteins forming RNA Induced Silencing Complexes (RISC). The RISC selectively degrades one of the siRNA strands, referred to as the passenger strand. The remaining strand, referred to as the guide strand is used to target the complementary sequence in mRNA molecules. Once the guide strand pairs with an mRNA in a RISC complex, the mRNA molecule is cleaved and degraded, preventing protein translation (Chau and Lee, 2007; Fire et al., 1998).

FSANZ have previously assessed potato event E12 (A1128), transformed with pSIM1278 and events W8, X17 and Y9, transformed with both pSIM1278 and pSIM1678 (A1139). FSANZ did not identify any potential public health and safety concerns with these events.

Details of a safety assessment of small RNA generated in Innate® potatoes was presented in A1139. According to scientific literature, RNA and siRNA are labile during processing and digestion and biological barriers further reduce potential exposure by limiting uptake of siRNA into the cells of mammals (Fabre et al., 2014; Hickerson et al., 2008; McAllan, 1982). There is no mechanism for harm in consuming siRNA in Z6 potatoes due to RNA lability during processing and digestion, extensive biological barriers that limit uptake and activity in cells, and the lack of complementarity between the potential siRNA in Z6 and the transcriptome and genome of humans. In addition, conservation of the potato *VInv* gene is limited to plants, providing additional evidence that potatoes containing small RNA directed against the potato *VInv* gene are as safe for consumption as conventional potatoes.

Based on the estimated exposure analysis, humans have very high margins of exposure for the siRNA consumed from Z6 tubers. Given that small RNA are ubiquitous in nature, present in all food, and unlikely to accumulate in the environment, consumption of Z6 potatoes and their associated siRNA is as safe as the consumption of conventional potatoes.

B.3(d)(i) where RNA interference has been used: the role of any endogenous target gene and any changes to the food as a result of silencing that gene

Glucose and fructose can accumulate in tubers during cold storage in a process known as cold-induced sweetening (Bhaskar et al., 2010). If potatoes contain high levels of reducing sugars, they can become undesirably dark and develop bitter flavours after frying (Halterman et al., 2016).

Four of the potato transcripts targeted for reduced expression are: asparagine synthetase, phosphorylase-L, water dikinase, and polyphenol oxidase. The aim of the suppression of asparagine synthetase is to reduce levels of free asparagine and the aim of suppression of PhL and R1 is to reduce levels of the reducing sugars, fructose and glucose. Collectively, the reduction of free asparagine and reducing sugars results in potato tubers with reduced acrylamide potential. Reduced expression of polyphenol oxidase results in tubers with reduced blackspot bruising. The down regulation of these genes has been assessed by FSANZ (Application 1128 and 1139).

The potato vacuolar invertase (VINV) is also targeted for reduced expression through RNAi, resulting in lower reducing sugars. Following starch breakdown in the amyloplast, glucose, glucose-6-phophsate, and maltose are transferred to the cytoplasm. From there, the sugars are further metabolised and shuttled into the glycolysis pathway for mitochondrial respiration or converted into sucrose (Malone et al., 2006; Sowokinos, 2001). Invertase enzymes including vacuolar invertase (VINV) hydrolyse the sucrose into glucose and fructose. Down regulation of VINV reduces the conversion of sucrose to fructose and glucose during cold storage, which results in lower levels of acrylamide upon frying and inhibits formation of sugar-related defects in fries and chips (Halterman et al., 2016; Ye et al., 2010).

Lower free asparagine and lower reducing sugars are within the range for conventional potatoes and are considered substantially equivalent to edible potatoes (Section B5).

B.3(d)(ii) where RNA interference has been used: the expression levels of the RNA transcript

The expression levels of transcripts from the 5 potato enzymes are presented in Section A.3(g).

The reduced expression of asparagine synthetase, polyphenol oxidase, and vacuolar invertase were consistent with the compositional and trait efficacy data in Sections B.1 and B.5. The inserted cassettes were successful in reducing expression of asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and vacuolar invertase transcripts in Z6 tubers. Down regulation of the targeted transcripts was not observed in Z6 leaves, stems, roots, or flowers.

B.3(d)(iii) where RNA interference has been used: the specificity of the RNA interference

The reduced expression of the 5 potato genes is facilitated by the *Agp* promotor of the ADP glucose pyrophosphorylase gene (*Agp*) and the *Gbss* promoter of the granule-bound starch synthase gene (*Gbss*). Both promotors are primarily active in tubers. The specificity of reduced expression is demonstrated in Section A.3(g). The inserted cassettes were successful in reducing expression of asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and vacuolar invertase transcripts in Z6 tubers. Down regulation of the targeted transcripts was not observed in Z6 leaves, stems, roots, or flowers.

B.5 Compositional analyses of the food produced using gene technology

This must include all of the following:

B.5(a) the levels of relevant key nutrients, toxicants and anti-nutrients in the food produced using gene technology compared with the levels in an appropriate comparator (usually the non-GM counterpart). A statistical analysis of the data must be provided.

B.5(b) information on the range of natural variation for each constituent measured to allow for assessment of biological significance should any statistically significant differences be identified

B.5(c) the levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator as well as the range of natural variation.

Compositional Assessment of V11 and Z6

Compositional analysis of Z6 [unpublished report] was conducted to evaluate the levels of key nutrients (proximates, vitamins, amino acids, and minerals) and glycoalkaloids compared to the non-transformed control variety Snowden. In addition, composition analysis is provided for the primary event V11 [unpublished report].

The compositional assessments evaluated:

1. Proximates, vitamins, and minerals (Table 13 and Table 14);
2. Total amino acids (Table 15 and Table 16); and
3. Glycoalkaloids (Table 17 and Table 18).

Analytes were measured from tubers harvested from field trials conducted for phenotypic and agronomic assessments. Additional details for the composition and statistical methods can be found in:

- [unpublished report]; V11
- [unpublished report]; Z6.

The analytes selected for the compositional assessment were based on the recommendations in the OECD consensus document on compositional considerations for new varieties of potatoes: key nutrients, anti-nutrients, and toxicants (OECD, 2002).

For V11, additional potato varieties were grown as reference material to provide a range of values common to conventional potatoes. The reference varieties are commonly used in the chip, fry, dehydrated, or fresh markets. Reference materials were used to calculate tolerance intervals. Tolerance intervals were used for compositional data to represent the natural variability among potatoes. The tolerance interval attempts to predict the range in which most values of a population will fall (Vardeman, 1992). The tolerance intervals were calculated to contain, with 95% confidence, 99% of the values in a population. The control varieties were used in the tolerance interval calculation because of their widespread popularity and history of safe use. The inclusion of the controls in the tolerance interval did not affect the statistical analysis because the tolerance interval was a separate calculation. For Z6, the combined literature range included the potato data from the ILSI crop composition database, so tolerance intervals were no longer used.

The compositional results from V11 were compared to this tolerance interval and the combined range of values for each analyte available from the published literature. The compostion results from Z6 were compared to the combined literature range. In interpreting the data, emphasis was placed on the analyte means. Means that fell within the tolerance interval and/or CLR for the analyte were considered to be within the normal variability of commercial potato varieties.

Detailed compositional analysis results for Z6, and V11 are described and summarised below.

Proximates, Vitamins, and Minerals

A statistically significant difference between V11 and the control was seen for vitamin C (Table 13). However, mean values for vitamin C were within the tolerance interval and combined literature range. These results indicate that V11 was equivalent to conventional potatoes. The remainder of the analytes presented in Table 13 showed no statistically significant difference between V11 and the control.

Table 13. Proximates, Vitamins, and Minerals in V11 and Snowden

Compound	Variety	Mean	P-value[1]	N[2]	Standard Deviation	Range		Tolerance Interval[3]		Combined Literature Range[4]	
						Min	Max	Min	Max	Min	Max
Moisture (%)	V11	78.5	0.1064	22	1.89	76.0	83.0	71.7	87.0	63.2	86.9
	Control	79.2		21	1.83	76.3	83.2				
Protein (%)	V11	2.34	0.9048	22	0.259	1.99	2.91	0.830	3.48	0.700	4.60
	Control	2.33		21	0.240	2.01	2.82				
Fat (%)	V11	0.166	0.8899	22	0.0530	0.100	0.300	0.100	0.500	0.0200	0.200
	Control	0.162		21	0.0610	0.100	0.330				
Ash (%)	V11	1.03	0.6646	22	0.105	0.820	1.20	0.500	1.37	0.440	1.90
	Control	1.01		21	0.107	0.803	1.20				
Crude Fiber (%)	V11	0.475	0.3731	22	0.0860	0.340	0.630	0.197	0.830	0.170	3.50
	Control	0.503		21	0.102	0.353	0.700				
Carbohydrates (%)	V11	17.9	0.1296	22	1.87	13.5	20.5	9.30	25.4	13.3	30.5
	Control	17.3		21	1.81	13.4	20.4				
Total Calories (kcal/100 g)	V11	82.5	0.1161	22	7.70	64.0	93.2	48.8	111	80.0	110
	Control	79.9		21	7.29	64.2	92.1				
Vitamin B_3 (Niacin) (mg/100 g)	V11	2.19	0.0984	22	0.259	1.62	2.64	0.794	2.68	0.0900	3.10
	Control	2.05		21	0.201	1.68	2.32				
Vitamin B_6 (mg/100 g)	V11	0.110	0.9855	22	0.0110	0.0970	0.140	0.0640	0.190	0.110	0.340
	Control	0.110		21	0.0110	0.0960	0.140				
Vitamin C (mg/100 g)	V11	26.9	**0.0050**	22	2.45	22.1	32.0	12.1	34.4	1.00	54.0
	Control	24.1		21	4.10	15.2	30.4				
Copper (mg/100 g)	V11	0.0800	0.9679	22	0.0230	0.0500	0.120	0.0500	0.160	0.0200	0.700
	Control	0.0800		21	0.0240	0.0500	0.120				
Magnesium (mg/100 g)	V11	22.6	0.2320	22	3.77	17.9	31.0	11.3	31.0	11.3	55.0
	Control	21.8		21	3.51	17.4	29.4				
Potassium (mg/100 g)	V11	488	0.1021	22	43.0	426	605	240	587	350	625
	Control	473		21	39.2	405	557				

[1]P-values indicating significant differences are underlined and in bold.
[2]Data from one replicate of the Snowden control from the Florida 2012 site were omitted because of an error at harvest.
[3]99% Tolerance Interval, 95% confidence.
[4]Literature ranges are from Horton and Anderson, 1992; Lisinska and Leszczynski, 1989; Rogan et al., 2000; Talburt et al., 1987.

Statistically significant differences between Z6 and Snowden were observed for carbohydrates, calories, moisture, Vitamin B3, and Vitamin C (Table 14). However, mean values for each of these analytes were within the combined literature range. These results indicate that Z6 was equivalent to conventional potatoes. The remainder of the analytes presented in Table 14 showed no statistically significant difference between Z6 and Snowden.

Table 14. Proximates, Vitamins, and Minerals in Z6 and Snowden

Variable	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Protein (%)	Z6	2.33	0.7562	0.174	16	2.11	2.73	0.7	4.6
	Snowden	2.31		0.149	16	2.08	2.61		
Total Fat (%)	Z6	0.158	0.1983	0.102	16	0.100	0.370	0.02	0.74
	Snowden	0.178		0.118	16	0.100	0.420		
Ash (%)	Z6	0.931	0.4871	0.0900	16	0.792	1.10	0.15	2.0
	Snowden	0.905		0.158	16	0.462	1.11		
Crude Fiber (%)	Z6	0.609	0.8192	0.0990	16	0.453	0.870	0.17	3.5
	Snowden	0.603		0.105	16	0.425	0.780		
Carbohydrates (%)	Z6	19.3	**0.0002**	2.41	16	16.2	23.3	3.68	29.4
	Snowden	18.1		2.56	16	14.5	24.5		
Calories (kcal/100 g)	Z6	87.4	**0.0006**	9.49	16	73.9	103	22.4	110
	Snowden	83.2		9.97	16	68.5	107		
Moisture (%)	Z6	77.4	**0.0003**	2.37	16	73.4	80.6	71.8	86.0
	Snowden	78.5		2.50	16	72.4	82.1		
Vitamin B3 (mg/100 g)	Z6	1.58	**0.0071**	0.243	16	1.28	2.05	0.88	3.43
	Snowden	1.46		0.275	16	1.15	2.00		
Vitamin B6 (mg/100 g)	Z6	0.142	0.0605	0.0130	16	0.121	0.160	0.065	0.204
	Snowden	0.133		0.0110	16	0.105	0.150		
Vitamin C (mg/100 g)	Z6	26.7	**0.0395**	3.03	16	21.5	31.2	6.97	51.4
	Snowden	24.8		3.01	16	19.7	30.1		
Copper (mg/100 g)	Z6	0.113	0.2273	0.128	16	0.0320	0.570	0.04	2.05
	Snowden	0.0831		0.0440	16	0.0250	0.160		
Magnesium (mg/100 g)	Z6	23.8	0.0809	2.10	16	20.1	28.2	14.6	40.6
	Snowden	22.6		2.20	16	20.0	29.4		
Potassium (mg/100 g)	Z6	479	0.1082	34.0	16	405	527	291	765
	Snowden	461		22.7	16	409	492		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges are from ILSI, 2019 and OECD, 2002.

Mean values of proximate, vitamin, and mineral levels were measured in V11 and Z6 tubers. Although statistically significant differences were observed in each event, the mean values of proximates, vitamins, and minerals were within the tolerance interval and/or combined literature range. Therefore, V11 and Z6 are nutritionally equivalent to conventional potatoes in proximates, vitamins, and mineral levels.

Total Amino Acids

Amino acid levels were measured in V11 and Z6 tubers (Table 15 and Table 16). Significantly lower aspartic acid + asparagine and significantly higher glutamic acid + glutamine were noted between V11 and Snowden and Z6 and Snowden. These results were expected because of the down-regulation of the *Asn1* gene in both V11 and Z6. The means for these analytes were within the CLR.

Statistically significant differences between V11 and Snowden were also noted for alanine, arginine, cystine (including cysteine), glycine, isoleucine, leucine, serine, threonine, tyrosine, and valine (Table 15). In all cases, the mean values for V11 were within the tolerance interval and/or the combined literature range, and therefore considered equivalent to conventional potatoes.

A significant difference between Z6 and Snowden was also noted for all other total amino acids, with the exception of histidine and tryptophan (Table 16). In all cases, the mean for Z6 was within the CLR, so Z6 was considered equivalent to conventional potatoes.

Statistically significant differences were observed in amino acid levels in each event. However, all mean values for total amino acids were within the tolerance interval and/or combined literature range. Therefore, the levels are within the normal range of compositional variation and not nutritionally meaningful. The events V11 and Z6 are equivalent to conventional potatoes in total amino acids.

Table 15. Total Amino Acids in V11 and Snowden

Compound	Variety	Mean (mg/100 g)	P-value[1]	N[2]	Standard Deviation	Range		Tolerance Interval[3]		Combined Literature Range[4]	
						Min	Max	Min	Max	Min	Max
Alanine	V11	70.9	**<u>0.0067</u>**	22	5.62	60.4	82.9	22.4	105	39.2	95.2
	Control	64.2		21	4.99	56.7	76.1				
Arginine	V11	142	**<u>0.0056</u>**	22	29.4	109	204	15.8	188	70.0	138
	Control	123		21	21.6	89.4	169				
Aspartic Acid + Asparagine[5]	V11	300	**<u><.0001</u>**	22	35.0	249	377	44.2	799	339	738
	Control	519		21	62.9	414	627				
Cystine	V11	30.2	**<u>0.0221</u>**	22	5.12	23.5	41.8	10.0	49.5	48.0	92.5
	Control	26.6		21	3.97	22.7	35.7				
Glutamic Acid + Glutamine[6]	V11	495	**<u><.0001</u>**	22	79.3	327	653	128	581	292	604
	Control	350		21	44.4	283	428				
Glycine	V11	72.7	**<u>0.0103</u>**	22	7.89	59.3	89.3	10.0	110	1.00	97.5
	Control	65.4		21	6.67	56.8	81.7				
Histidine	V11	36.0	0.1944	22	5.74	30.1	49.1	11.5	52.5	13.3	46.9
	Control	34.3		21	5.14	27.5	45.7				
Isoleucine	V11	82.2	**<u>0.0085</u>**	22	9.05	67.7	101	20.0	123	52.5	95.3
	Control	75.5		21	8.37	63.8	94.5				
Leucine	V11	138	**<u>0.0026</u>**	22	13.0	114	167	10.0	225	68.5	138
	Control	124		21	11.5	109	153				
Lysine	V11	118	0.0534	22	11.4	99.8	143	36.6	173	68.7	137
	Control	111		21	8.76	102	132				
Methionine	V11	39.2	0.1648	22	4.03	31.8	46.6	11.3	59.7	9.00	128
	Control	36.9		21	3.52	30.2	42.9				
Phenylalanine	V11	96.6	0.0638	22	10.7	75.9	121	11.7	154	55.2	109
	Control	91.2		21	9.73	76.6	114				
Proline	V11	78.9	0.3559	22	16.1	55.8	111	10.0	155	35.5	146
	Control	72.3		21	13.7	51.9	95.3				
Serine	V11	82.7	**<u>0.0049</u>**	22	10.2	63.2	103	10.0	130	50.0	102
	Control	74.7		21	7.30	62.0	90.9				
Threonine	V11	85.6	**<u>0.0027</u>**	22	8.91	70.3	105	11.5	129	43.6	85.5
	Control	77.7		21	7.53	68.6	97.1				
Tryptophan	V11	20.9	0.2731	22	4.66	13.9	32.2	10.0	36.3	11.4	28.2
	Control	20.1		21	4.47	11.5	27.6				
Tyrosine	V11	85.9	**<u>0.0020</u>**	22	10.2	72.0	108	17.3	124	45.7	94.2
	Control	76.1		21	8.83	66.1	94.3				
Valine	V11	109	**<u>0.0225</u>**	22	13.0	90.0	133	43.3	159	75.2	145
	Control	102		21	12.2	82.6	123				

[1]P-values indicating significant differences are underlined and in bold.

[2]Data from one replicate of the Snowden control from the Florida 2012 site omitted because of an error at harvest.

[3]99% Tolerance Interval, 95% confidence. Negative values or values below the limit of detection, arising from variability measured in the samples, were adjusted to the limit of detection (10 mg/100 g).

[4]Combined literature ranges are from OECD, 2002; Rogan et al., 2000; Talley et al., 1984.

[5,6]Reported as total aspartic acid plus asparagine and total glutamic acid plus glutamine. During analysis, an acid hydrolysis step converts asparagine to aspartic acid and glutamine to glutamic acid, respectively.

Table 16. Total Amino Acids in Z6 and Snowden

Variable	Variety	Mean (mg/100g)	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Alanine	Z6	74.8	**<u>0.0007</u>**	8.4	16	61.6	89.6	10.0	145
	Snowden	62.5		5.13	16	54.8	69.7		
Arginine	Z6	147	**<u>0.0015</u>**	17.4	16	124	192	46.2	234
	Snowden	127		11.4	16	114	153		
Aspartic Acid + Asparagine	Z6	308	**<u><.0001</u>**	27.4	16	270	364	177	1548
	Snowden	502		50.0	16	408	594		
Cystine	Z6	33.8	**<u>0.0003</u>**	3.97	16	26.6	38.9	10.0	125
	Snowden	27.6		3.71	16	20.5	33.3		
Glutamic Acid + Glutamine	Z6	510	**<u><.0001</u>**	42.4	16	426	584	152	956
	Snowden	369		27.8	16	326	429		
Glycine	Z6	79.2	**<u><.0001</u>**	8.63	16	67.8	91.9	30.7	372
	Snowden	66.1		5.94	16	57.4	76.2		
Histidine	Z6	38.9	0.0658	4.93	16	32.8	50.0	10.0	105
	Snowden	35.2		4.33	16	29.1	43.7		
Isoleucine	Z6	86.0	**<u>0.0047</u>**	9.53	16	73.1	103	21.3	137
	Snowden	74.7		6.43	16	65.4	85.4		
Leucine	Z6	148	**<u>0.0004</u>**	18.9	16	124	181	53.0	224
	Snowden	120		11.2	16	104	140		
Lysine	Z6	124	**<u>0.0033</u>**	15.2	16	103	147	44.4	495
	Snowden	107		9.38	16	92.9	120		
Methionine	Z6	39.5	**<u>0.0009</u>**	3.47	16	34.2	46.7	10.0	83.6
	Snowden	34.7		2.55	16	30.3	39.4		
Phenylalanine	Z6	101	**<u>0.0065</u>**	11.2	16	85.7	121	41.4	131
	Snowden	90.3		7.51	16	78.4	104		
Proline	Z6	80.9	**<u>0.0005</u>**	8.55	16	70.4	98.7	31.9	232
	Snowden	67.8		6.79	16	58.2	79.2		
Serine	Z6	87.9	**<u>0.0007</u>**	10.5	16	74.1	104	10.0	140
	Snowden	75.7		7.00	16	66	86.9		
Threonine	Z6	94.2	**<u>0.0028</u>**	10.6	16	80.2	111	19.8	133
	Snowden	79.2		7.63	16	69	90.9		
Tryptophan	Z6	23.1	0.0582	1.97	16	19.7	25.9	10.0	32.1
	Snowden	22.0		1.72	16	18.7	24.8		
Tyrosine	Z6	89.4	**<u>0.0008</u>**	10.3	16	76	109	27.5	237
	Snowden	73.7		6.34	16	63.5	83.1		
Valine	Z6	109	**<u>0.0166</u>**	9.85	16	97.7	131	24.6	259
	Snowden	99.0		9.86	16	86.5	122		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges are from ILSI, 2019 and OECD, 2002.

Glycoalkaloids in V11 and Z6

Glycoalkaloids are toxins commonly found in Solanaceous crops, including potato, and 95% of the total glycoalkaloids in potato tubers consists of α-solanine and α-chaconine (OECD, 2002).

The mean concentration of glycoalkaloids was not significantly different between V11 and the control. In both varieties the mean concentration was lower than the safety limit, and fell within the tolerance interval and the combined literature range (Table 17). The safety limit for total glycoalkaloids in tubers is 20 mg/100 g fresh weight (Smith et al., 1996).

The mean concentration of glycoalkaloids was significantly different between Z6 and Snowden. However, in both Z6 and Snowden, the mean concentrations were lower than the safety limit, and fell within the combined literature range (Table 18).

Table 17. Glycoalkaloids in Tubers from V11 and Control Snowden

Compound	Variety	Mean (mg/100 g)	P-value[1]	N[2]	Standard Deviation	Range		Tolerance Interval[3]		Combined Literature Range[4]	
						Min	Max	Min	Max	Min	Max
Glycoalkaloids[5]	V11	9.70	0.3878	22	4.10	5.00	19.4	5.00	20.4	3.20	210.4
	Control	10.8		21	7.21	5.04	38.9				

[1]P-values indicating significant differences are underlined and in bold.
[2]Data from one replicate of the Snowden control from the Florida 2012 site were omitted because of an error at harvest.
[3]99% Tolerance Interval, 95% confidence.
[4]Combined literature ranges from Kozukue et al., 2008.
[5]Total of α-solanine and α-chaconine.

Table 18. Glycoalkaloids in Tubers of Z6 and Snowden

Variable	Variety	Mean (mg/100g)	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Glycoalkaloids[3]	Z6	11.8	**0.0439**	3.16	16	7.26	19.0	3.20	210.4
	Snowden	13.9		3.70	16	9.08	23.5		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges from Kozukue et al., 2008.
[3]Total of α-solanine and α-chaconine.

Patatin in V11 and Z6

Potatoes are not among the “Big Eight” group of foods that account for ~90% of all food allergies in the U.S. (FARRP, 2014). There are a few reports of allergies to cooked potato in children (DeSwert et al., 2002, 2007). However, most children with potato allergy develop tolerance at mean age of four years (De Swert, et al., 2007). Patatin (Sol t 1) has been identified as the primary allergen involved in this reaction (Astwood et al., 2000). Patatin is a storage glycoprotein that displays lipase activity and makes up about 40% of the soluble

protein in tubers (Mignery et al., 1988). There is no mechanistic reason to suggest that the level of patatin would be changed in V11 or Z6. Because potato protein naturally contains a relatively large proportion of patatin, any unexpected change in patatin levels would be unlikely to affect allergenicity enough to alter consumption patterns for people allergic to potatoes.

Conclusion of the Compositional Assessment of V11 and Z6

A thorough compositional assessment of key nutrients, including proximates, vitamins, minerals, and amino acids, and of glycoalkaloids was conducted for V11 and Z6. Statistically significant differences were observed in the levels of some proximates, vitamins, minerals, and amino acids in V11 and Z6. However, the observed levels were within the tolerance interval and/or combined literature range, demonstrating that the nutritional content of V11 and Z6 falls within the normal range for potatoes. These results demonstrate that V11 and Z6 are compositionally equivalent to Snowden and to other commercial potato varieties.

Glycoalkaloid levels in V11 and Z6 were unchanged and within the recommended safety limits. Because potato protein naturally contains a relatively large proportion of patatin, any unexpected change in patatin levels would not affect allergenicity enough to alter consumption patterns for people allergic to potatoes. The compositional assessment demonstrated that V11 and Z6 are as safe and nutritious as conventional potatoes with a long history of safe consumption.

Low acrylamide potential and lowered reducing sugars

An assessment of V11 and Z6 for low acrylamide potential and lowered reducing sugars consisted of the following analyses.

1. Free amino acids in tubers (Table 19 and Table 20)
2. Reducing sugars in tubers (Table 21 and Table 22)
3. Acrylamide in chips (Table 23 and Table 24).

Free amino acids

Acrylamide is a chemical that can form when certain starchy foods are cooked or processed. While there's no direct evidence that acrylamide can cause cancer in humans, there is evidence it can cause cancer in laboratory animals (FSANZ 2016[6]).

Acrylamide forms during cooking and processing predominately from the reaction of free asparagine and reducing sugars in the Maillard reaction. Events V11 and Z6 have been developed to have reduced levels of asparagine.

Free amino acids in V11 Potatoes

The free amino acid analysis demonstrated that down-regulation of *Asn1* was effective in reducing free asparagine in tubers. The results show that V11 tubers contained significantly less free asparagine and significantly more free glutamine than Snowden tubers (Table 19). However, the mean concentrations of free asparagine and free glutamine for V11 were within the tolerance interval and the combined literature range and therefore considered within the normal range for potatoes.

[6] http://www.foodstandards.gov.au/consumer/chemicals/acrylamide/Pages/default.aspx ; Retrieved August 2019

Table 19. Free Amino Acids in Tubers from V11 and Control Snowden

Compound	Variety	Mean (mg/100 g)	P-value[1]	N[2]	Standard Deviation	Range		Tolerance Interval[3]		Combined Literature Range[4]	
						Min	Max	Min	Max	Min	Max
Asparagine	V11	79.4	**<.0001**	22	21.6	35.5	128	10.0	520	31.2	689
	Control	312		21	51.4	212	407				
Aspartic Acid	V11	53.7	0.3054	22	35.0	33.8	77.8	4.20	71.4	6.4	75.2
	Control	51.5		21	62.9	35.8	74.0				
Glutamine	V11	222	**<.0001**	22	62.2	71.2	322	10.0	298	44	539[5]
	Control	125		21	36.0	55.9	181				
Glutamic Acid	V11	66.5	0.2872	22	13.5	37.9	90.2	4.40	96.4	45	74.2
	Control	61.8		21	11.5	41.9	78.4				

[1]P-values indicating significant differences are underlined and in bold.
[2]Data from one replicate of the Snowden control from the Florida 2012 site were omitted because of an error at harvest.
[3]99% Tolerance Interval, 95% confidence. Negative values or values below the limit of detection, arising from variability measured in the samples, were adjusted to the limit of detection (100 mg/100 g).
[4]Combined literature ranges are from (Davies et al., 1977; Lisinska and Leszczynski, 1989; Shepherd et al., 2010)
[5]A value of 1,824mg/100 g from a single site in the combined literature range was not included because it appeared to be an outlier.

Free amino acids in Z6 Potatoes

Analysis demonstrated that Z6 tubers contained significantly less free asparagine and significantly more free glutamine than Snowden tubers (Table 20). However, the mean concentrations of free asparagine and free glutamine for Z6 were within the combined literature range and therefore considered within the normal range for potatoes. Free amino acid analyses demonstrated that down regulation of asparagine synthetase was effective in reducing free asparagine in tubers.

Table 20. Free Amino Acids in Tubers of Z6 and Snowden

Variable	Variety	Mean (mg/100 g)	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Asparagine	Z6	80.4	**<.0001**	14.8	16	55.4	104	31.4	456
	Snowden	309		44.1	16	237	397		
Aspartic Acid	Z6	45.1	0.5897	3.85	16	39	51.7	16.7	197
	Snowden	44.2		3.60	16	37.3	50.5		
Glutamic Acid	Z6	57.6	0.061	8.28	16	44.6	71.7	12.5	136
	Snowden	54.5		7.83	16	44.1	68.7		
Glutamine	Z6	259	**<.0001**	40.1	16	186	314	33.6	411
	Snowden	162		22.1	16	118	193		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges are from ILSI, 2019.

Reducing Sugars

Down regulation of water dikinase and phosphorylase slows the breakdown of starch into sugars in the amyloplast resulting in lower levels of fructose and glucose reducing sugars. The down regulation of invertase slows the breakdown of sucrose into glucose and fructose in the vacuole, resulting in decreased levels of glucose and fructose and increased sucrose levels in tubers. Sucrose, glucose and fructose levels were measured in events V11 and Z6 and Snowden at harvest and for V11 after storage at 10°C temperatures.

Reducing Sugars in V11 Potatoes

V11 showed a trend for lower levels of the reducing sugars, fructose and glucose, although the differences were not statistically significant (Table 21). V11 was designed to lower levels of reducing sugars in tubers by slowing the breakdown of starch into sugars in the amyloplast. Mean results for fructose plus glucose and sucrose in V11 were within the tolerance interval and the combined literature range for both fresh and stored conditions.

Table 21. Sugars in Tubers from V11 and Control Snowden at Harvest and After Storage at 10 °C

Timing	Variety	Mean	P-value[1]	N[2]	Standard Deviation	Range		Tolerance Interval[3]		Combined Literature Range[4]	
						Min	Max	Min	Max	Min	Max
Fructose + Glucose (mg/100 g)											
Fresh[5]	V11	26.7	0.7689	22	31.1	5.50	108	1.00	435	18	803
	Control	35.1		21	46.2	5.20	145				
Month 3[6]	V11	53.5	0.2127	6	74.2	11.5	204	1.00	435	18	803
	Control	151		5	137	26.7	319				
Month 6[7]	V11	39.4	0.9450	3	0.212	11.5	95.0	1.00	435	18	803
	Control	14.7		3	4.06	11.1	19.1				
Month 9[7]	V11	92.3	0.9970	3	9.93	80.9	99.1	1.00	435	18	803
	Control	105		3	20.2	84.2	125				
Sucrose (mg/100 g)											
Fresh[5]	V11	197	0.8569	22	90.5	114	424	1.00	443	39.7	1,390
	Control	194		21	97.4	116	432				
Month 3[6]	V11	147	0.4911	6	13.4	131	170	1.00	443	39.7	1,390
	Control	179		5	62.1	127	262				
Month 6[7]	V11	98.0	0.7371	3	62.0	55.0	169	1.00	443	39.7	1,390
	Control	74.2		3	10.4	62.4	82.1				
Month 9[7]	V11	171	0.9867	3	33.5	146	209	1.00	443	39.7	1,390
	Control	145		3	2.65	143	148				

[1]P-values indicating significant differences are underlined and in bold.
[2]Data from one replicate of the Snowden control from the Florida 2012 site were omitted because of an error at harvest.
[3]99% Tolerance Interval, 95% confidence.
[4]Literature Ranges from Amrein et al., 2003; Vivanti et al., 2006.
[5]Samples analyzed at the fresh time points were from all 2012 and 2013 field trial locations.
[6]Samples analyzed after three month storage were from all 2012 field trial locations.
[7]Samples analyzed after six and nine month storage were from Montcalm County, Michigan 2012.

Reducing Sugars in Z6 Potatoes

Z6 showed significantly lower levels of reducing sugars, fructose and glucose, at harvest (Table 22). These results can be attributed to partial down regulation of R1 glucan water dikinase, and down regulation of phosphorylase L and vacuolar invertase. Down regulation of R1 glucan water dikinase and phosphorylase L slows the breakdown of starch into sugars in the amyloplast.

Sucrose levels were significantly higher in Z6 at harvest compared to Snowden (Table 22). This difference can be attributed to the down regulation of invertase, which slows the conversion of sucrose into fructose and glucose in the vacuole. However, mean sucrose content for Z6 was within the combined literature range for potatoes at harvest.

Table 22. Sugars in Tubers of Z6 and Snowden at Harvest

Variable	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Fructose and Glucose (mg/100 g)									
Fresh	Z6	6.76	**0.0517**	2.12	16	4.02	10.1	13	1208
	Snowden	17.7		12.7	16	7.52	55		
Sucrose (mg/100 g)									
Fresh	Z6	133	**0.0036**	16.7	16	109	161	39.7	1390
	Snowden	122		16.7	16	91.3	151		

[1]P-values indicating significant differences are underlined and in bold.
[2]Literature Ranges from Amrein et al., 2003; Vivanti et al., 2006.

Summary of Reducing Sugars in V11 and Z6

As expected, compared to Snowden levels of reducing sugars in V11 and Z6 were lower at harvest and after storage, confirming the efficacy of the lower reducing sugars trait. For fresh Z6, the mean reducing sugar levels were below the literature range for conventional potatoes, which is expected given the intended trait. The mean concentrations of sucrose were within the combined literature range and therefore within the normal range for potatoes.

Acrylamide Potential

Although acrylamide is not present in fresh potatoes, it is formed when the amino acid asparagine and the reducing sugars glucose and fructose are heated at high temperatures. Therefore, lowering the concentrations of free asparagine, glucose, and fructose reduces the acrylamide potential of cooked potatoes.

To determine the effect of lower asparagine and lower reducing sugars in V11 and Z6, acrylamide levels were measured in:

- Chips made from field-grown tubers of V11 and Z6 at harvest and for V11 after storage at 10 °C.

The significantly lower levels of free asparagine and reducing sugars resulted in lower acrylamide potential in V11 and Z6.

Acrylamide Potential of V11

At the time of harvest, chips made with V11 tubers contained 64.3% less acrylamide than chips made with Snowden. When potatoes were stored for three, six, and nine months at 10 °C, acrylamide concentrations in chips made from V11 were reduced by 48.9, 47.9, and 15.6%, respectively (Table 23).

Acrylamide concentrations in V11 chips were significantly lower than Snowden at the time of harvest and after three months storage. The significantly lower acrylamide levels after storage were expected from down-regulation of the *Asn1, R1 and PhL* genes, which lowered free asparagine and reducing sugars. Similar reductions in reducing sugars and acrylamide were reported by Zhu et al., 2014.

Lowered free asparagine, fructose and glucose levels lead to an overall reduction of acrylamide in processed potato products because they are reactants in the formation of acrylamide.

Table 23. Acrylamide in Chips from V11 and Snowden at Harvest and After Storage at 10 °C

Timing	Variety	Mean (ppb)	P-value[1]	Percent Reduction	N[2]	Standard Deviation	Range	
							Min	Max
Fresh[3]	V11	262	**<.0001**	64.3	22	127	112	540
	Snowden	734			21	414	239	1,540
Month 3[4]	V11	289	**0.0066**	48.9	6	186	125	582
	Snowden	566			5	206	399	857
Month 6[5]	V11	306	0.6386	47.9	3	28.1	279	335
	Snowden	587			3	217	337	717
Month 9[5]	V11	708	0.9839	15.6	3	323	499	1,080
	Snowden	839			3	270	530	1,030

[1]P-values indicating significant differences are underlined and in bold.
[2]Data from one replicate of the Snowden control from the Florida 2012 site were omitted because of an error at harvest.
[3]Samples analyzed at the fresh time points were from all 2012 and 2013 field trial locations.
[4]Samples analyzed after three month storage were from all 2012 field trial locations.
[5]Samples analyzed after six and nine month storage were from Montcalm County, Michigan 2012.

Acrylamide Potential of Z6

At harvest, chips made with Z6 tubers contained 77.8% less acrylamide than chips made with Snowden (Table 24).

Acrylamide concentrations in Z6 chips were significantly lower than Snowden chips at harvest. The significantly lower acrylamide levels were expected from down regulation of asparagine synthetase, R1 glucan water dikinase, phosphorylase L and vacuolar invertase, which reduced the free asparagine and reducing sugar reactants. Similar reductions in reducing sugars and acrylamide were reported by Zhu et al., 2014.

Table 24. Acrylamide in Chips from Z6 and Snowden at Harvest

Variable	Variety	Mean (ppb)	P-Value[1]	Standard Deviation	N	Percent Reduction	Range	
							Min	Max
Fresh	Z6	334	**<u>0.0168</u>**	94.7	8	77.8%	191	464
	Snowden	1,506		355	8		998	2,150

[1]P-values indicating significant differences are underlined and in bold.

<u>Summary of Acrylamide Potential in V11 and Z6</u>

The significantly lower levels of free asparagine and reducing sugars resulted in lower acrylamide potential in V11 and Z6. Although the down regulation of *R1* and *PhL* was less effective, the intended trait of lower reducing sugars is still prevalent in these events from the down regulation of *VInv*. Similar reductions in reducing sugars and acrylamide due to the down regulation of *VInv* were reported by Zhu et al., 2014.

C. Information related to the nutritional impact of the genetically-modified food

Potato has a long history of safe use. Global production in 2017[7] was in excess of 388 million tonnes. Two thirds were consumed directly by humans and the remaining fed to animals or used to produce starch.

The V11 and Z6 events in this submission have been transformed with T-DNA designed to down-regulate endogenous potato genes and in Z6 to produce the VNT1 protein. The introduction of the RNAi sequences and the VNT1 protein have no nutritional impact on the potato events. This is supported by the fact that:

- Molecular characterisation demonstrated stability of the inserts during vegetative propagation cycles
- The VNT1 protein has homologues with a history of safe consumption and no significant homology to known allergens and toxins; and
- Compositional analysis did not indicate biologically significant changes to the levels of nutrients in events compared to their conventional counterparts. Event composition is within the normal variation of potato cultivars and varieties and is substantially equivalent to conventional potato varieties.

The most important nutritional changes between V11 and Z6 potatoes and their untransformed controls, relate to reduction of the amino acid asparagine (ASN) and reducing sugars, as well as the expression of a late blight resistance protein VNT1. Thus, food products derived from V11 and Z6 potatoes are anticipated to be nutritionally equivalent to food products derived from other commercially available potatoes, except that V1 or Z6 potatoes cooked at high temperatures are expected to have lower acrylamide.

D. Other Information

Where a biotech food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies will add little to the safety assessment and generally are not warranted (see e.g. Bartholomaeus et al., 2013; Herman and Ekmay, 2014; OECD, 2003).

The only new polypeptide produced by the inserts in potato event Z6 is VNT1. This protein has a non-toxic mode of action and occurs at very low levels in the plants. Its safety is supported by a weight-of-evidence that indicates safety for human consumption. Considering the compositional equivalence between each potato event and its conventional variety, and the lack of any observed phenotypic characteristics indicative of unintended effects arising from the genetic modification process, there was no plausible risk hypothesis that would indicate the need for animal feeding studies.

The regulatory agencies in the United States and Canada have not required feeding studies for Z6 potatoes.

[7] Food and Agriculture Organization of the United Nations, http://faostat3.fao.org; data retrieved 18th October 2019.

Reports

[unpublished report] Compositional Assessment of V11 Compared to Snowden

[unpublished report] Expression of RNAi Targeted Transcripts in Snowden V11

[unpublished report] Characterization of *Rpi-vnt1* expression in Y9

[unpublished report] Z6 Insert Characterization by Southern Blotting

[unpublished report] Allergen and Toxin Evaluation of Open Reading Frames in Z6

[unpublished report] VNT1 Protein and *Rpi-vnt1* Gene Expression in Z6

[unpublished report] Stability of Inserts in Vegetatively Propagated Z6

[unpublished report] Sequence Characterization of the Inserts in Z6

[unpublished report] Compositional Assessment of Z6 Compared to Snowden

[unpublished report] Efficacy of Reduced Polyphenol Oxidase Activity in Z6 Tubers compared to Snowden at Harvest and After Storage

[unpublished report] Expression of RNAi-targeted Transcripts in Z6

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